Master Thesis

Fluorescent Microscopy with Adaptive Optics
Deep Tissue Wavefront Estimation for Sensorless Aberration Correction

Date : September 27, 2015

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Za Mamu i Tatu ...
Preface, Caution and Thanking

Data manipulation makes the result less straightforward but can arrange the authors to show only one selected part. Toward which extent does this reflect a scientific demarch is a subjectif appreciation. It remains part of some certain form of fine tuning until a solution following a logical flow appears and a prove that it is actually logical. The author does not necessarily agree with the form.

This thesis summarises a team job where everyone lead a task of predilection but merging onto other arms was from far not excluded. Before diving into the heart of the topic, I would like to thank every single person that I met during the process of this Thesis.

Xiaodong Tao (/chinese characters missing/), from him and his ability to quickly and calmly think and fasten I have an admiration hill. Tuwin, Dare worked on the physical alignment of the 3 Photon scope that has unfortunately not been tested yet because of the Cazadero’s Laser delivery issue? . Ramiro and Qinggele (Dk. Li) the software control interface for the deformable mirrors. J. Lu from the admirable Y. Zuo’s Lab for the biological support (will not be included in the thesis). Marco and Joanah for making me recover part of my antecedent grounded perspectives. And finally, Professor Kubby, for the devotion he shows toward the healthy functioning of his group, in such an art that allowed me a wide freedom. There could not have been a supervisor from whom I would have learned more at my stage. Thanks dudes! Oh and Prof. Richard Hahnloser for still accepting my thesis.

My part was focusing on the estimation and prediction of the system’s aberrations. It is not to repeat that the following document is not exclusively from me and I take all the responsibility
with the best honesty. Maybe before continuing, let me add here a memorial on the 5 mice (not any acknowledged further) that have been spared (in accordance with all the Rights) for the following piece.
Abstract

The multiple light scattering in biological tissues limits the measurement depth for traditional wavefront sensor. The attenuated ballistic light and the background noise caused by the diffuse light give low signal to noise ratio for wavefront measurement. To overcome this issue, we introduced a wavefront estimation method based on a ray tracing algorithm. With the knowledge of the refractive index of the medium, the wavefront is estimated by calculating optical path length of rays from the target inside of the samples. This method can provide not only the information of spherical aberration from the refractive index mismatch between the medium and biological sample but also other aberrations caused by the irregular interface between them. Preliminary results are demonstrated.
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Preface, Caution and Thanking

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In the first place, images happen (accidently) in our mind and give an impulse to our mind …Next after this comes the will

Titus Lucretius Carus

De rerum natura

Pushing frontiers of two competing facets in awake animals imaging: Highest spatio image resolution and deepest tissue imaging. Two concepts with a common main limitation argument: Scattering. Adding to those two concepts temporal resolution and we can seek to identify functional properties of biological tissues. This thesis won’t cope with any biology. Instead, the technical goal is to seek for resolution and deepness that could be applied on a question for the synaptic level¹.

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¹And dynamics with calcium imaging, as the technique was well driven here [19] but data analysis did not reveal some repetitive feature from which a law could emerge
CHAPTER 1. INTRODUCTION OF THE PROBLEMS IN CLASSICAL MICROSCOPY

Let me come back to the measuring device and introduce it by presenting the limitations, diverse technique as counteracts and their performance, but first, the ground base of microscopy.

Figure 1.1: Minimal Microscope Model

Focused beam passing through a lense will transform from spherical to planar wave. Lenses are in a conjugate plane with the pupil plane when the distance between them and the different optical elements is equal to the focal length. Fig. 1.1 shows, from left to right, the sample on the object plane at the focal distance of the objective lens ($O_L$). Next comes the aperture, at a focal distance $O_L$ as well, and further behind, the tube lens ($T_L$) at its focus length followed by the image plane. All elements from an optical table are aligned to stay in conjugate plane with the pupil. The magnification $M$ is given by $M = \frac{T_L}{O_L}$.

1.1 Scattering and the Diffraction Limited Problem

The aperture\(^2\) acts as a slit and interferes with light. A spherical wave can be modelled by complex exponentials whose elements within the same phase (Wavefront, WF) group onto a sphere. Each point of the wave can itself be seen as a source of spherical wave [5]. The exponentials are sine and cosine functions whose amplitude are constrained by the superposition principle. The projected result, on an image plane for example, will produce fringes. The radius of the Airy’s disk [12] is given by the distance between the center of the aperture and the first minima (or intersection of the chief ray, for non isotropic scenarios (broken spherical symmetry) with the plane and the first minima).

The Airy Disk constitutes the diffraction limit\(^3\). Its radius is proportional to the wavelength (main reason why electron miscoscope have much better resolution , but have, to my knowledge, never been applied on living organisms\(^4\)) and inversely proportional to the diameter of the aperture.

---

\(^2\)see Fig. 1.1 for the position of the aperture in an objective. No part is dedicated on the necessity of the aperture

\(^3\)In litterature, usually proportional to the wavelength over the image-pupil disance (not cited). We did not go into verifications

\(^4\)Samples have to be coated with a conductive material and only the surface can be imaged because scattering is inversely proportional to the wavelength, see [33] and further
Before continuing any further, I’ll quickly expand this problem by introducing another important concept in optics and in particular in Fourier Optics: The Point Spread Function (PSF).

The PSF is the 3D equivalent of the Airy Disk, including the paraxial dimension. The PSF is the spatial meeting of rays around the focus point. In finite and isotropic cases, the PSF takes the shape of an ellipsoid whose paraxial semi axis reaches (from above) the Diffraction Limit.

Going beyond the diffraction limit was done either by introducing sequentially fluorescent molecules that would be activated sparsely and then inactivated by bleaching and the next subset would be activated and so on. Localization would be achieved by looking at the center of the point spread function [2]. Another way, less constrained but more sophisticated is to use structured illumination but I could not find any study that achieved the limit in this way.

E.g. Optical tomography (modulating frequencies of coupled lasers allows, by superposition problem, to modulate the diffraction and recover some information by doing a convolution of the image with the point spread function for example[6]) is a theme that can be expanded by a laborious algebra and reconstruction algorithms to adapt it for the field of fluorescence microscopy[7].

We will leave aside the question on whether it is possible to resolve at the size of a point.

Scattering in turbid media decreases with longer wavelength [8]. This gives advantage for using visible light. And even more credit for increasing the number of photons for excitation [26]. The disadvantage is the higher light intensity that is then required[8]. Indeed, more photons need to hit the fluorescent protein at the same spatio temporal location and be absorbed for an event to occur. The event’s probability is therefore decreased.

### 1.2 Aberrations and Optical Path Difference

In this part, we will focus on Monochromatic and Chromatic aberrations. I am not sure if all aberrations can be encompassed by only those two categories. It must be said that there are different orders of aberrations. What will be considered and called ‘low order’ consists mostly of refraction of ballistic light (ruled by Snell’s law 1.2). High order as reflection will not be treated here at all. It could have potentially merged us toward tomography. In [32], multiple scattered

---

5I think the aperture assures this condition
6Within the PSF, the aberrations seem to be somehow correlated?.
7The idea was proposed by my colleague Marc Reining for his PhD dissertation
8Have to use femtosecond pulsed lasers in multi-photon configurations, to avoid photobleaching the sample whereas in confocal microscopy, a continuous illumination is sufficient
light is filtered out using temporal light detection\(^9\).

**MONOCHROMATIC**

Those aberrations (piston, tilt, defocus, astigmatism, coma, spherical, trefoil, etc.) are well spanned by the Zernike Polynomials. See details in Annexe A. We will use the polynomials when we will be discussing about wavefront corrections in § 3.1

Monochromatic aberrations such as defocus and spherical are responsible for the PSF elongation in the \( z \) propagation or paraxial direction. Many sensorless techniques, based on trigonometric formulations of the problem have been employed to predict and correct them: [23], [22], [24]. Brute Force, Genetic Algorithm and Hill Climbing have been respectively proposed in [27], [20] and [7] to handle all aberrations. Beside the temporal complexity of the search algorithms (more than a half of a minute is needed to correct, not suited for live corrections), their application remain constraint only on the specific treated problem and do not translate much of the physics behind.

**CHROMATIC**

Dispersion causes the chromatic aberrations. The refractive index being wavelength dependent\(^{10}\), different light colors will follow a different bent through mismatched refractive index medias (prism effect) and therefore focus on different spots, widening the PSF. This matter has to be taken into account especially in multi-photon adaptive optics where different energy scales for emission and for excitation occur. We will discuss about this issue in sections 4.1.1 and 4.2.2, when we will be talking about measuring aberration coming from emission and correction is performed on excitation (such is the case in our design, see § 2.1)\(^{11}\). To our knowledge, no scheme that embrace the chromatic aberrations has been brought yet.

**OPTICAL PATH DIFFERENCE**

\[
\text{OPD} = l_1 n_1 - l_2 n_2
\]  

(1.1)

The optical path difference (OPD) for a beam travelling through one interface separating two medias with refractive index \( n_1 \) and \( n_2 \) is given by the difference of the optical path lengths (OPL) that the light would have had without interface. The numerical aperture (NA) defines

\(^9\)time-gated detection; photons that scatter multiple times come back later to the detector. An interferometer records the optical path difference between the reference (laser source reflected off by a beamsplitter) and a reconstruction algorithm selects single scatterers. There is no mention on intensity of information loss

\(^{10}\)see Cauchy or Sellmeier empiric equations for more details

\(^{11}\)The impact of this effect has been neglected in [4]
1.3. PERFORMANCE OF AN OPTICAL SYSTEM

the base of the cone of light at the aperture (unitless), it gives the relation between the incident angle of marginal rays and the refractive index. Snell’s law assure that this is independ of the media and through his equation, either next marginal angle\textsuperscript{12} or RI can be inferred:

$$\text{NA} = n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$$  \hspace{1cm} (1.2)

To get the phase displacement, multiply equation (1.1) by $\frac{2\pi}{\lambda}$, where $\lambda$ stands for the wavelength of the propagating beam. The phase difference due to flat, chief-ray-orthonormal mutli medias interfaces refletes in spherical and defocus aberrations (see section Results in 4.1). This can be confirmed by computing the Zernike coefficients of the phase difference. The non zero coefficients correspond to piston, defocus and sphercial order aberrations.

Equation (1.1) generalizes to an arbitrary $N$-layered system:

$$\text{OPD} = l_1 n_1 - \sum_{i=2}^{N} l_i n_i$$  \hspace{1cm} (1.3)

Aim reside in identifying the different OPLs in vacuum, given by $l_i$ and the corresponding RIs. As an application we would like to consider the system microscope-brain as a 3 layers system\textsuperscript{13}. To calculate the optical path delay induced by different refractive index layers, the rays are traced from the source (inside the tissue) and substracted to the unperturbed path (a sphere). We will come back with more details on this issue in 3.1.1. Fig. 1.2 is very inspired from [23]. The figure does not account for defocus (It is separatly removed from this group’s simulations) but gives the essence for 1 interface.

1.3 Performance of an Optical System

\textsuperscript{12}I think, by numerically decreasing NA, we can access the bending information for all rays ranging from marginal to paraxial. We will be considering this when we will be estimating the position of the focus point in 3.1.1

\textsuperscript{13}This approximation is a rather crude assumption. Dura matter can be added and even further consider the different brain layers. Map the various tissues with their RIs and account as much shape information as possible
We will not go too much in the calculations of Fourier Optics (this would likely merge us in the field of tomography again (usefull when more than one laser enters the system) but will rather focus on maximizing the pixel intensity appearing on the monitor and on minimizing the root mean square of the aberrations (of the Wavefront) which in turn is assumed equivalent (but less extendible) than the previous idea.
Chapter 2

Fluorescence Microscope with Adaptive Optics

Except for the roulette wheel, everything required for the Project had been homemade

Thomas A. Bass
The Eudaemons

Adaptive Optics using similar devices as the one presented and used in this chapter can produce images at the diffraction-limited resolution among the almost entire 72 hours young Zebrafish [3].

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2.1 Two Photon Optical Design, as Global Overview

Fig 2.1 shows a diagram of the experimental apparatus that we used. Let me present the excitation and emission light paths.

Excitation or illumination light is emitted by a pulsed\(^1\) Ti:sapphire. The collimated laser beam is modulated through the deformable mirror (DM), described in § 2.1.2, before it enters the sample.

\(^1\)Drawback of pulsed laser resides in their wider frequency range. The shorter the pulse, the longer the dispersion [28], to imagine via Fourier Transform
through the water immersion objective lens (25x). A scanner\(^2\) allows the beam to be moved in a maximal window of 200\(\mu\text{m}\) \(\times\) 200\(\mu\text{m}\).

The light that is emitted by the sample is reflected through an amovible dichroic mirror (DB1) that transmits longer wavelength such as excitation and reflects lower wavelength such as emission into a Photomultiplier Tube (PMT\(^3\)). Another filter (not shown here) can be placed in front of the PMT to narrow the range to the emission spectrum by cutting the even lower wavelength (e.g. to wash out autofluorescence). The photomultiplier is necessary because, although emission is usually in the visible domain, there are not enough events for our vision to detect it.

A Shack Hartman Wavefront sensor (SHWS), described in § 2.1.1, senses the light that is emitted from the sample. For this, DB1 is temporarily removed from the light path and a second, permanent dichroic mirror (DB2) ensures that the visible light will be reflected toward the SHWS, to capture an image of the aberrations.

The sample lays on an XYZ motorized stage.

\subsection{2.1.1 Direct Wavefront Sensing}

The Shack Hartmann sensor, (most common AO-sensor), measures the gradient of the wavefront. The gradient (localized slope) is, by computation, translated in phase difference (directly proportional to the optical path difference, as mentioned earlier in § 1.2). We refer to section 3.1 for the direct sensing method. The Shack Hartmann sensor used here consists of 11x11, (focus length:24cm) lenses (lenslets) arranged in a rectangular grid. An EMCCD camera is placed in the focal plane of the lenslets. Using the shifts of the focal point \(\Delta x\) and \(\Delta y\) in the sensor plane, and knowing the focal length of the lenslets permit to calculate the gradient of the wavefront.

Some of the whole transparent worm larve neuronal activity (calcium) has been measured with a similar device, the light field camera \[29\].

Sensorless method will be described in § 3.1.

\subsection{2.1.2 Wavefront Correcting}

The wavefront correcting tool is the heart of an adaptive optics set up and cannot be avoided. Many devices can be used (simultaneously). The correcting devices usually have a maximal range of operating system that they can apply for. Partitionning aberrations between multiple devices

\(^2\)Not very suited for dynamical imaging (video)
\(^3\)This device is responsible for the sampling density (or digitalization)
has been done here [35], [24] [6] [36] to bring the front of the wave to its aberrationless state, i.e planar WF.

**Deformable Mirror**

The Deformable Mirror (DM) with 12x12 (in our case) actuators displacing locally the mirror’s (continuous, in our case) surface changes the phase of the light by the double of the displacement, reshaping thus the WF. Our actuators have only the freedom of piston (moving back and forth). There exist a segmented version, where actuators can also tilt. Each DM needs to be calibrated. Usually the voltage to be applied on the actuators are not linear with the displacement (to overcome some electric force). Roughly said, a voltage is applied on each actuator separately and the so created aberration is measured giving rise to a control relation (a matrix, see section 3.1 for wavefront modulation details).

To calibrate a DM, voltage is applied on one actuator and the generated aberration is then measured with the WF sensor. This is performed across all the actuators and for many voltages, building the direct relation matrix. We briefly come back on the matrix when describing the modulation of the DM in methods in § 3.1.

**2.1.3 Spatial Light Modulator and Digital Micromirror Device**

Since we are not using those two tools but they constitute a redundant part in AO, I’ll quote them in two lines each.

A spatial light modulator is usually a crystal whose refractive index can locally be modified and therefore affects the refracted light phase.

Digital Micromirror Device grids thousands of mirrors that can be switched on and off (by flipping). They are suited for live correction [36] but intensity might be lost by half if half of the mirrors are flipped off.

**2.2 (Bio)-luminescence**

It is not understood which cell and why those ones are going to express the FP. Stochasiticy is said to govern the labelling intensity, the expression pattern. Types and numbers of labelled neurons varies greatly among mice. Different colors tend to accumulate in different regions of the cell (e.g. Red FP in the nucleous) [11]. Another intriguing effect, on the control of the targeting of various fluorescence proteins is known through the brainbow [9], [34].
2.2. (BIO)-LUMINESCENCE

We will mostly use microsphere YPF : Emission 527 nm Excitation 960nm. [25]
the Galaxy is nothing else but a mass of innumerable stars planted together in clusters

Galileo Galilei
The Astronomical Messenger

P**recision is the key**, from polishing the first lens to the current objectives (most precious device consisting the microscope), passing through alignment and soft/hardware calibration.

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Beam lights on the $12 \times 12$ DM’s actuators (crossed arrows) and $11 \times 11$ Shack-Hartmann’s lenslets (dots).

3.1 WaveFront Corrections

Let $x, y$ be a set of coordinates in the pupil plane. $z$ is along the beam propagation.

Any Wavefront $W(x, y)$ can be decomposed in a series of Zernike Polynomials $Z_i(x, y)$, up to an arbitrary order $N$:

$$W(x, y) = \sum_{j=1}^{N} c_j Z_i(x, y)$$  \hspace{1cm} (3.1)

The principle resides in bringing the Wavefront’s aberration back to the unaberrated configuration. The Zernike Polynomials lend themselves as good intermediates to convert phase shifts into voltages to be applied on the DM. Half of the mirror’s displacement corresponding to the phase shift (for the back and forth of the light path).

Recall, the polynomials are depicted in appendix A. The four first modes corresponding to piston, $x, y$ tilt and defocus. Those four modes are not corrected by the DM. Piston represents a
3.1. WAVEFRONT CORRECTIONS

translation of the Wavefront in the z-plane and tilt in the xy-plane. Those two transformations do not affect the shape of the PSF neither the relative displacement of the WF sensor's lenslets, instead, the whole image is translated (by approximatively the tilted angle times the distance to the image). The refocusing is rather done by moving the stage in the z direction instead of using the DM. The DM typically has a certain ammount of aberration that it can correct for and an overvoltage (above 200V, in our setup) will result in a irreversible break.

DIRECT CORRECTION

Image of the emission light passing through the lenslets is taken with the WF sensor. Our technique presents drawback as to know what the lenslets focus shift is based on. The reference is visually selected with a mask that seems aberrationless (i.e. when the spacement between the lenslets focus appears as a regular grid). Subsequent shifts will be taken in accordance to the chosen modell\(^1\). We will discuss the reference issue in 4.1.

The extraction of the Zernike coefficients from lenslets focus shift is a linear procedure and best described in [18]. Here we give the idea:

\[ c = Z^+ s \] (3.2)

The \(2K\) spots displacement \(\Delta = (\Delta x_1, ..., \Delta x_K, \Delta y_1, ..., \Delta y_K)\) recorded with the EMCDD camera at the focal plane of the \(K\) lenslets are in pixel. An algorithm returns the shifts (from the previously selected mask) of the centroids of the spots based on their intensity. Knowing one pixel's size, the conversion can be done in any length unit. If not specified, results will be renormalized to have final units of red wavelength (\(\lambda = 633\)nm).

The gradient or slope is obtained by dividing the displacements by the focus length of the lenslets \((f_l = 2400\, \text{mm})\) \(s = \frac{\Delta}{f_l} = (s_{x1}, ..., s_{xK}, s_{y1}, ..., s_{yK})\).

From calibration, a control matrix \(A\) is constructed that translates the sensitivity of the Wavefront Sensor to the deformable mirror and computes the actuator command, in voltage

\[ v = A^+ s \] (3.3)

\(^1\)For accuracy, knowing the lenslets spacement should allow the creation of the perfect reference, incrementing on x and y, i.e i times spacemenet j times spacemenet gives the theortetical reference
Tilt is filtered from the slope vector by subtracting the global (mean) tilt \( \overline{s_x} = \frac{\sum_{k=1}^{K} s_{kx}}{K} \) and \( \overline{s_y} = \frac{\sum_{k=1}^{K} s_{ky}}{K} \) and piston is removed on the voltages:
\[
\overline{v} = \frac{\sum_{m=1}^{M} v_m}{M}
\]
Combining (3.2) and (3.3) gives the following formula that derives the voltages from the Zernike Coefficient only.

\[
v = A^+ Z c
\]  

(3.4)

**INDIRECT, SENSORLESS**

Aberrations not only prevent the surviving ballistic photons from forming a diffraction-limited focus, degrading signal, imaging contrast and resolution [3] [17] but in deep measurement, they prevent emission light to shine on the wavefront sensor. The intensity decreases with depth and depending on the sample, the wavefront sensor can’t sense anymore. On our scope, mouse brain, the signal on the wavefront sensor is lost at the depth limit circa 600\(\mu m\) in vivo and beyond 1800 \(\mu m\) in clarity (for clarity sample preparation, see [31]). In the oil toy sample (see § 4.2), the limit was achieved at 349\(\mu m\).

Equation (3.4) is a good basement for a sensorless method, since it is independent from the slope of the lenslets.

Knowing the medium in which the light propagates back out of the tissues, it should be possible to completely predict the aberrations due to scattering at an order where probabilistic nature of quantumness is neglected, at least.

**3.1.1 The formalism of ray tracing**

The problem dealt with in this note may be formulated in this way: Imagine first-order ray tracing\(^2\) in the sense that it does not include many of the known physics but follows fundamental principle of the older physics, namely the principle of deterministic causality.

In this note, rays are traced sequentially, treated in the same sequence as a rescaled time. The rays follow the Fermat principle in euclidian space and, known the initial position and direction, the most important information is, in this pre given order, encaptured at the intersection between two interfaces.

\(^2\)A problem very well known in Computer Graphics [13]
3.1. WAVEFRONT CORRECTIONS

It is a common problem in computer graphics; knowing the intersecting position between a ray under certain physical laws and a given object. We present the ensuing method, called ray tracing, to assess the wavefront aberrations. Here is the statement:

Rays are traced from two adjacent surfaces in a predefined sequence. The interface between the surfaces could be any arbitrary type of surface. With a single layer, such as water in case of water immersion objective, the light from the objective forms a perfect spherical wavefront and focus at the point $O_o$. From this point, a perfect reference sphere can be constructed with radius $R^3$. Then the ideal OPL of each ray from $O_o$ to the reference sphere through the single medium can be determined by:

$$\text{OPL}_{\text{ideal}} = n_M R$$

where $n_M$ is the RI of the top layer (the one in contact with the objective lens). The ray from the target is defined in spherical coordinate as

$$I(\theta, \phi) = \begin{pmatrix} \cos \theta \sin \phi \\ \sin \theta \sin \phi \\ \cos \phi \end{pmatrix}$$

where $\theta$ and $\phi$ are azimuth and zenith angle (radius is normalized). Then the optical aberration is calculated as the optical path difference between the ideal OPL and the real OPL of ray between the target and the reference sphere,

$$\text{OPD}(\theta, \phi) = \sum_{j=1}^{M} n_j I_j - \text{OPL}_{\text{ideal}}$$

where $n_j I_j$ is the OPL of the ray in the medium $j$ and $n_j$ it’s RI. The final WF in the pupil plane $W(r, \phi')$ can be calculated from Eq. 3.6 by converting the spherical coordinate at the target point to polar coordinate at the pupil plane. Suppose the ray $I(\theta, \phi)$ from the target intersects the reference plane at $P(p_x, p_y, p_z)$. Then the coordinate of ray in the pupil plane is given by

$^3R$ is the focus of the objective lens in the immersion medium, $R = f = \frac{D}{NA}$, with $D$ the diameter of the aperture.
\[ r = \sqrt{p_x^2 + p_y^2} \]  
\[ \phi' = \arctan(p_x p_y) \]  
(3.8)

where \( p_x \) and \( p_y \) are \( x \) and \( y \) coordinates respectively. The origin \( z \) is taken at the center of the reference sphere (the nominal focus point if no RI mismatched interfaces are present).

\[ P_k = P_{k-1} + dI_k \]  
(3.9)

The intersection point with a surface is calculated by replacing in the surface parametrization the equation for the rays () and solving for \( d \).

Two main unknowns that need to be determined for the ray tracing formalism to be usable. The first one is the refractive index and the second is the depth in tissue.

Notations are adapted from the bible of computer graphism [10].

### 3.1.2 The depth in tissue, geometrical optics issue

The position of the source is unknown. We used similar formel as [1]. The PSF being principally elongated in \( z \), where should the reference be taken?\(^4\)

We will always refer \( d \) as the stage displacement in the last surface (in tissue) and \( t \) the actual depth of the focus point in the last surface. \( \theta_1 \) is the angle in the immersion water and \( \theta_2 \) the angle in the second interface, then:

\[ t = d \frac{\tan \theta_1}{\tan \theta_2} \]  
(3.10)

Taking into account the power laser distribution mostly gaussian, we suppose that the main intensity is given by the paraxial rays and trim NA.

\(^4\)An alternative is given by the natural propretie of energie minimazion. Wavefront optimization was done either by minimizing the root mean square of the wavefront or minimizing the Zernike coefficients. Both revealed to be equivalent, because of the non uniform power distribution (either from laser or apodization from the aperture). To improve the model, a Gaussian quadrature scheme [16] emphasizing the paraxial rays could have been used. The solution presented by geometrical optics seemed more direct.
One, three, two: one, three, two.
It was all very curious

Fiodor Dostoïevsky
*the Gambler*

To demonstrate the wavefront estimation in optical microscopy using the ray tracing method we will focus on tri-layered samples: the tissue (to be specified), a cover glass and the objective water immersion (NA=1.05). Labels are YPFs, recall:

- **Excitation**: 525 nm
- **Emission**: 960 nm
- **Normalization**: $\lambda=633$ nm

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4.1 Glass Coverslip and Microscope Collar

The sample is a borosilicate coverslip with certified thickness of 0.17 ± 0.005 mm. Yellow fluorescent microparticles, or more simply beads of 1 µm diameter were first diluted in Acetone. (10% solution, for sparsity) and swept on both surfaces of the coverslip (measured thickness, with a calipers (±25 µm) is 0.173 mm). After drying, the borosilicate coverslip is placed on a drop of water on a wafer and eventually sealed.

The reference mask (seen from the outcome of the lenslets on the EMCCD camera : Second Fig. 4.2) is taken at the top as the collar ring is set to zero. Ten measurements are performed on the top and bottom of the coverslip, at diverse locations. The shift of stage, in order to focus at the bottom of the glass varies is 46 µm. The aberrations are depicted in 4.1 where values from the top (fluctuating a bit around zero) are subtracted to the values taken just below. (There are some small fluctuations around zeros, ±0.1λ, for dominant aberrations at the top of the glass and larger, ±0.2λ (e.g defocus) fluctuations at the bottom). See 4.1 for the mean outcome.

As today, most of the objectives are equipped with a collar ring (z-amovible system of lenses) capable of correcting spherical and defocus aberrations due to a flat and beam light orthonormal glass coverslip, this correction has to be subtracted from our modell (to avoid as much as possible overloading the DM, and tend toward a device operating at maximal efficiency). In

\[\lambda\]

Zernike Mode

Figure 4.1: Sensed aberrations due the coverslip

---

1For further experiment, the thickness of the glass should be measured with a micrometer, much more precise, as well
order to assess the contribution of the collar ring, the ring is set at 0.17 mm and the image is
taken by focusing on beads put on the top of the coverslip. Left Fig. 4.2 shows the aberrations
induced by the ring. The negative contributions of the ring should be very close to the earlier
measured aberrations, at the bottom of the glass. There is a mismatch between the objective
collar induced aberrations at the top and the measurement at the bottom in this configuration.
Especially for the defocus $Z_4$. This can be because the glass is thicker than 0.17mm and/or
because the mask already captures some of the positive aberrations and does not correspond to
a flat wavefront (mean defocus and spherical aberrations are reported in the caption below).

$$Z_4 = -1.96\lambda$$  
$$Z_{11} = -0.66\lambda$$

$Z_{mask} = 0.885\lambda$  
$Z_{flat} = 0.622\lambda$

Figure 4.2: Wavefront Sensor Output

Fig. 4.3 gives the direct wavefront measurement for the lenslets’ configurations of the most left
and right images of 4.2, following procedure described in § 3.1 (Defocus is deducted for the plot,
since the DM does not correct it). Defocus adds a half of an ellipsoid to the wavefront, see
the Zernike Polynomials $A$. Positive defocus implies that the paraxial rays are focused closer
to the lens than the focal length and they bend toward the outside when exiting the objective
(collimation is lost), resulting in a convex wavefront.

### 4.1.1 Refractive Indexes of the Glass

**EMISSION RI**

We will use, as reference, the mean defocus generated from the bottom of the coverslip obtained
in the previous set of measurements, i.e. $Z_4 = 0.88\lambda$ to get the glass RI, for the emission wave-
length. The three interfaces are water-glass-water with the water’s emission RI taken at 1.3342
Two schemes for searching are proposed:

- Either the algorithm searches over depth in tissue \( t \) and RI. The prediction gives RI=1.52 and a depth in tissue \( t = 2.2 \mu m \). See Fig. 4.4.

- Or, geometrical optics (§ 3.1.2) can resolve for depth in tissue, given the stage displacement, and the search is then only performed over RI. The prediction gives also RI=1.52 but a depth in tissue \( t = 0.028 \mu m \). See Fig. 4.5.

The both predicted depths do not match. The expected one is 0.5\( \mu m \), since the bead has 1\( \mu m \) of diameter we can expect to be focusing in the middle. This will be discussed in § 4.4

\(^2\)http://refractiveindex.info/?shelf=main&book=H2O&page=Hale
4.1. GLASS COVERSILP AND MICROSCOPE COLLAR

Since the correction is performed on the illumination light, we need to know the excitation RI. Using water’s excitation RI of 1.3270\(^3\), this is done by minimizing the WF at the depths obtained before (and at the expected depth). Results are reported in Fig. 4.6. The best prediction is given

\(^3\)http://refractiveindex.info/?shelf=main&book=H2O&page=Hale
by optimizing the wavefront when focusing on the expected half bead, supposing either that the geometrical optics formula is not accurate enough or that the RIs of water are not compatible or the tracing algorithm not complete. This will also be discussed in § 4.4.

![Figure 4.6: Borosilicate Excitation RI](image)

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<tr>
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<th>Emission (960nm)</th>
<th>Excitation (525nm)</th>
</tr>
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<tr>
<td>Eperiment</td>
<td>1.52</td>
<td>1.49</td>
</tr>
<tr>
<td>Source</td>
<td>1.5199</td>
<td>1.5081</td>
</tr>
</tbody>
</table>

In the following results, the aberration due to the coverslip will be substracted from the code and the collar ring is set at 0.17µm

### 4.2 Beads in Oil

The sample consists of an unknown rather viscous oil (could be castor oil) poured in a limited recipient with depth of approximately 1.5mm. Beads are merged inside the sample and the aforementioned coverslip seals the whole. Wavefront measurement is performed at different stage displacement. The aberration evolution is shown in Fig. 4.7. The depth \( d \) correspond to the working distance shift from the first occurrence of the oil and deeper (Recall that 143 µm are needed to overpass the cover glass of 173 µm).

Fig. 4.8 is the image taken at the surface of oil. Fig. 4.8a has no adaptive optics. Fig. 4.8b uses the wavefront sensor as described previously and Fig. 4.8c comes from the sensorless method. No refocusing is done after correction.
4.2. **BEADS IN OIL**

Figure 4.7: $m(Z_4) = 175 \lambda /\text{mm}$, $m(Z_{11}) = 16.66 \lambda /\text{mm}$

![Graph showing Zernike Mode vs. d [µm]](image)

Figure 4.8: Beads at the surface of oil, Scale bar: 25µm

(a) No AO  (b) Sensor  (c) Sensorless

Fig. 4.9 compares the intensity of the green channel of RGB accross the line (laser intensity remains unchanged). The sensorless method reaches pixel saturation of the RGB scale. Since this is not very useful, comparison is done at the edge of the sensor’s and further at the edge of the DM.
4.2.1 Sensor Limit

The limit of the Wavefront sensor in Oil occurs at a depth of 349 µm. There is not enough light and some spots disappear (upper left corner of Fig. 4.10). Beyond this depth, it is impossible for the centroid algorithm to figure out the spot displacements.

Left column of Fig. 4.11 compares No AO (top) with the direct sensing, middle (S) and the sensorless (SL) bottom. The right column zooms the square from the first column. All the images are taken without refocusing except (S2) where the best focus was found by moving the \( z \) stage. In order to perform a fair comparison, best focus should be calculated for each type of correction.
4.2. BEADS IN OIL

(not done).

Figure 4.11: At the edge of Sensitivity

\[ d = 349 \, \mu m \], scale bar : 25 \, \mu m
Fig 4.12 compares the intensity across the line. Mostly the sensor method with the best focus is taking over.

4.2.2 Refractive Indexes of the Oil

Figure 4.13: Likelihood of the Error on Defocus
EMISSION RI

For the search over the RI, the reference from the wavefront sensor is taken at 10μm depth and a defocus of $Z_4 = 0.889\lambda$ (Fig. 4.7). The emission refractive index for glass is taken at 1.52 and this time the search is performed over the tissue’s RI and depth. Results suggest that the depth in tissue is 8μ and the emission RI for oil 1.54 is (see Fig. 4.13).

Whereas performing the search using the depth from geometrical optics, we find $RI \approx 1.425$ and a depth of 20.1 μm (see Fig. 4.14).

The expected depth should be longer than the working distance shift (recall: 20 μm). The refractive index of oil is in both cases larger than water, implying, from Snell’s law a narrower angle and therefore a longer penetration depth. But the glass as intermediate makes the prediction too simplified and not valid.

The error on the defocus is much smaller when the search is also performed over the depth.

Figure 4.14: Oil Emission RI, and predicted depth (top) in μm

EXCITATION RI

Minimizing the wavefront using the excitation RI for glass of 1.50 did not lead to any minimum (see Fig. 4.15). The reason is not clear. Whether it is a parametric mistake in the code or an inaccuracy in the depth of the stage (10μm?) or a model that simply does not include enough of the physics. The outcome of geometrical optics (20 μm) is not trustable since it has not been
tested on diverse samples.

4.2.3 Beyond the sensing limit; at the DM limit

This time we pushed the limit until the maximally allowed voltage for the DM. Beyond this depth, the predicted aberrations would be too large to be covered by the DM’s range. Fig. 4.16 compares the outcome without AO (Fig. 4.16a) with the sensorless method (Fig. 4.16b). I don’t remember if I searched for the best focus after correction, probably not.

After correction, new structures emerge (encircled) but some structures also disappear (crossed) on the right Fig. 4.16b. This is due to the focal shift. There is a coupling between spherical and defocus aberrations. Their polynomial is similar and by correcting spherical, a part of defocus is also affected.

4.2.4 Oil Drop

This section lights up the coupling between the SA and defocus. Fig. 4.17 is the image of an oil drop. Bottom is a z-scan. Top left is the surface of the bubble and top right is taken at a stage shift of $d=13 \, \mu \text{m}$ (No AO here). By using the sensorless method on the left state, very similar image as the on the right is obtained and the inside is better resolved (the image has not been saved unfortunately).
4.2. BEADS IN OIL

The shape of the bubble could improve the correction by including the surface normal angle to the incident beam, into Snell’s law Eq. (1.2). The oil bubble is an extendible problem to biological samples as embryos.
4.3 Clarity

The procedure done for oil is redone on a clarity brain. The aberrations evolution is in Fig. 4.18. We tried to fit the slope of the SA and use the outcome to correct at further depths but it was not sure from the result (not shown) if there was an overcorrection or if the best focus was to be tuned. We finally did not try to get the RI of clarity.

4.4 On the validation of the method: Discussion and further improvements

Putting beads on the top of the coverslip is subject to controversy. Beside that it might pollute the scope, they luminate much more than biological samples and might induce light pollution on
4.4. ON THE VALIDATION OF THE METHOD: DISCUSSION AND FURTHER IMPROVEMENTS

the image. Using YFPs or RFPs did not seem to change the quality of the image on either fixed or clarity\textsuperscript{4} tissues and I could not tell if there was more or less light pollution with or without the beads on the top. Beads could be completely avoided if the part of the working distance that accounts for the coverslip is known in advance. We could use previous observations (e.g. the 146 \( \mu m \) to overcome coverslips of 173 \( \mu m \)) or geometrical optics (first need to check on other glass samples with various thicknesses to see if the calculation is general) or wavefront optimization (where does the excitation focus?). Supposing we can have access to \( d \) for a glass of arbitrary thickness (the window glass used on live mouse was 150 \( \mu m \) thick), once the first fluorescent element occurs on the monitor, the new displacement shift is recorded and the difference with the predicted shift for the coverslip would account e.g. for the dura matter (in live brain). If there is no difference, it means that the tissue is touching the bottom of the glass. Autofluorescence was thought about to create a map of the surface of the tissue and fit it with polynomials before using the ray tracing algorithm. Or if there is no autofluorescence, simply use the first transients of the FPs to record the contour lines of the surface. In order to focus at \( t=1mm \) deep in a material, the area to be covered should have a radius of \( \approx n\theta t \approx NA \approx 1mm \). A program as ImageJ can stitch this area. This is performed for different depth. And the transient pixel’s position would provide shape information. This has been tested and one obstacle was found for entirely black fields where ImageJ does not know how to stitch them. This could be mastered by using the position of the stage. And further, having a map of the surface could help retrieving location in depth, for time evolutionary images.

Apodization shifts further the focusing plane (adding more defocus), is not taken into account (using Zemax optics studio, the shift would be about 5%). Laser Power distribution for the model on depth. Where does the maximal light intensity focus? The PSF’s density could not only help retrieving this information but also improve the image quality by convoluting it with the image.

Hard to validate if the predicted aberrations are real from our set of experiments (flat surfaces). Since any spherical aberration that is smaller to the defocus (until which extend is not known) will contribute to corrections. Setting beads on the top of the coverslip and shifting the stage further (increasing the spacement between the objective and the sample) and recording the defocus gives very similar results to the prediction (see Fig. 4.19).

\textsuperscript{4}Fixed mouse brain: The vertebra column is dislocated, the head cut with scissors, the brain removed and quickly dumped in a 5% Paraformaldehyde (PFA) solution. By passive diffusion, in this case, the surface (maybe up to 1mm) of the brain absorbs some of the PFA

\textsuperscript{5}for clarity sample preparation, see [31]
Figure 4.19: Measured and Predicted Defocus
Initially I wanted to light up that learning is improved in a chaotic (enriched) environment. But as this seemed not to be feasible, in the amount of time I gave myself (after the craniotomy), Mice apparently need one month to be actually valid for biological research and training would maybe take another one to get it to respond in the desirable way. This biological subject is relevant since from what I remember, it is the diversity (the change) in stimuli that causes new firing and therefore affects the synaptic plasticity\(^1\).

Learning efficiency or at least synaptic dynamics might be increased if during the task a multitude of rewarding objects are presented. In order to confirm this more, the mice should be trained in Enriched Environments (EE) and compared with standard cages. And then, the precision should be tuned to find out what are the changes, maybe at the neurotransmitter’s level.

The method could potentially go beyond 1mm (see limit in Oil § 4.2.1) and allow us to check whether new spines also emerge at this depth [21]. The microscope should be optimized to cover as much aberrations as possible and the resulting contribution substraced from the code. The method for assessing the refractive index is not mastered. The problem might issue from the laser power distribution, some ponderation could emphasize rays closer to the pupil center. The refractive index does not have the highest impact on the aberrations. The most sensitive parameter is the shift of the working distance. It must be sure that the measurement is accurate. Another method, not fully tested nor included reside in calculating the RI using the slope of the defocus or SA, by creating a look-up tables.

To resume on the beyond of the ‘information limit’, is it possible to focus on a dot? The answer of Quantum Mechanical Resolution Limits is that it cannot ignore the finite size of the particle. But what if we measure indirectly using some coupling or force between the particles? Unendliche

\(^1\)not fully related but in a discussion concerning America, it came about a study on mice in a paradise for mice that tend to drink less of the poison than the one living with choice only between water or poison. I could not believe nor find the study.
Magnetfelddichte? A stroke in this direction was performed in [14]. It could maybe also explain how sharks locate in the ocean.
The Zernike polynomials (see equations (A.1) and (A.2)) form a continuous orthonormal set over the unit circle. For further properties on the Zernike coefficients, we refer to [30], the reference of this chapter. Figure A.1 gives the plot of the polynomials.

\[ Z_n^m(\rho, \varphi) = \begin{cases} R_n^m \cos(m\varphi) & \text{if } m \geq 0 \\ R_n^m \sin(m\varphi) & \text{if } m < 0 \end{cases} \tag{A.1} \]

\( n \) is the highest radial order and \( m \), for the azimuthal degree of freedom, is an integer such that \( m \leq n \). The radius \( \rho \) is the renormalized pupil’s radius, \( \varphi \) is the azimuthal angle and \( R_n^m \) is a normalization factor:

\[ R_n^m(\rho) = \sum_{k=0}^{\frac{n-m}{2}} \frac{(-1)^k (n-k)!}{k!(\frac{n+m}{2} - k)!(\frac{n-m}{2} - k)!} \rho^{n-2k} \tag{A.2} \]

Figure displays up to the 15\textsuperscript{th} polynomial. Not all the polynomials are explicitly written and the normalization factor as well as the Zernike coefficient are omitted.
Figure A.1: 15 first Zernike Mode grouped with respect to the radial order.
Appendix B

Code

1 clear syst
2 \% Input shape of the brain and local position
3 \% Output wavefront, \textit{Zernike} coefficients and Voltage
4
5 \% \( L[1] \): Aperture->Glass \((n_{\text{Water}}=1.33)\)
6 \% \( L[2] \): Glass->Dura \((n_{\text{Glass}}=1.54)\)
7 \% \( L[3] \): dura->Brain \((n_{\text{Water}}=1.33)\)
8 \% \( L[4] \): Brain \((n_{\text{Brain}}=1.3526)\)
9
10 syst.wl=0.633e-3;
11 syst.resolution=41;
12 syst.NA=1.05;
13 syst.n=[1.3270 1.5081 1.3270]; \% Excitation
14 %syst.n=[1.3342 1.52 1.3342]; \% Emission
15 syst.gl=0.173;
16 syst.wd=0.14765;
17
18 \% Get depth in tissue given the stage displacement
19
20 deepness=get_depth_GO(syst)
21 \([c_{zk},\text{PlotZ},\text{PlotX},\text{PlotY})=\text{Wavefront}(syst,\text{deepness})\)
22 fataloutput=Voltage(syst,c_{zk},d)

Get depth in tissue given the stage displacement
APPENDIX B. CODE

depth_of_stage=syst.wd;

n=zeros(1,length(nn));% Rays are traced from top to bottom
for i=1:length(nn) % ...
    n(end-i+1)=nn(i); % (Permutation of the RI vector..)
end

theta=zeros(1,length(n));o=zeros(1,length(n)-1);d=o;∆=o;
d(1)=syst.gl; % here, just do vector of depth to generalize to n-int
theta(1)=asin(NA/n(1)); % Marginal ray at int-1
o(1)=depth_of_stage*tan(theta(1)); % Semi cross section on int-1
for i=2:length(n)
    theta(i)=asin(n(i-1)*sin(theta(i-1))/n(i)); % Snell
    ∆(i-1)=d(i-1)*tan(theta(i));
    if i<length(n)
        o(i)=o(i-1)-∆(i-1);
        d(i)=o(i)/tan(theta(i+1));
    end
end
depth_in_tissue=d(end);

for the OPL: (generate the rays, calculates intersection new direction on arbitrary number of interfaces and finally the optical path length

function [c_2k,Plot2,PlotX,PlotY]=Wavefront(syst,deepness)
NA=syst.NA;Resolution=syst.resolution;wl=syst.wl;
n=syst.n;gl=syst.gl;depth_of_stage=syst.wd;
load('../spherical153.mat')
LOC=cell(1,length(n)+1);
DIR=cell(1,length(n));
r=3.99; % Ref sphere
thickness=r-depth_of_stage;
%
LOC{1}{1:Resolution^2} = deal([0 ;0 ;r-thickness-gl-deepness]);
DIR{1}=RayGen_DIR(NA,n{1},Resolution); % Starting Directions
O_sphere=[0; 0; 0]; % Flat surfaces
N{:,1}=[0 ;0; 1];
angle=0; % Add tilt
N{:,2}=[-sind(angle) ;0; cosd(angle)];
\(\begin{align*}
N(:,3) &= [-\sin(\text{angle}) \; 0 \; \cos(\text{angle})] ; \\
\text{depth}(1) &= r - \text{thickness} - gl - \text{deepness}; \quad \% \text{source} \\
\text{depth}(2) &= \text{depth}(1) + \text{deepness}; \\
\text{depth}(3) &= \text{depth}(2) + gl; \\
\% \text{ray tracing} \\
\text{Int3} \text{matrix} &= \text{zeros}(\text{Resolution}^2, 3); \\
\text{for} \; i = 2: \text{length}(n) \\
\text{LOC}(i) &= \text{cellfun}(\oplus(x,y) \; \text{Plane} \; \text{LOC}(N(:,i), \ldots \\
& \quad \text{depth}(i), x, y), \text{LOC}(i-1), \text{DIR}(i-1), 'uni', 0); \\
\text{n_r} &= n(i-1)/n(i); \\
\text{DIR}(i) &= \text{cellfun}(\oplus(x) \; \text{Refracted} \; \text{DIR}(n_r, N(:,i), x), \text{DIR}(i-1), 'uni', 0); \\
\text{end} \\
\% \text{The last intersection, with the sphere:} \\
[\text{LOC}(i+1), -] &= \text{cellfun}(\oplus(x,y) \; \text{Sphere} \; \text{LOC}(r, O_{\text{sphere}}, x, y), \text{DIR}(i), \text{LOC}(i), 'uni', 0); \\
\text{path} &= \text{zeros}(1, \text{length}(n)); \text{tot} = \text{zeros}(1, \text{Resolution}^2); \\
\text{for} \; i = 1: \text{Resolution}^2 \\
\quad \text{for} \; j = 1: \text{length}(n) \\
\quad \quad \text{path}(j) &= \text{norm}(\text{LOC}(j-1) - \text{LOC}(j+1)*n(j); \\
\quad \quad \text{end} \\
\quad \text{tot}(i) &= \text{sum}(\text{path})/wl; \\
\quad \text{end} \\
\% \text{Uncomment for figure of the rays} \\
\% l &= \text{cellfun}(\oplus(v) \; v(i), \text{LOC}); \\
\% \text{linel} &= [l{:}] ; \\
\% \text{figure}(1) \\
\% \text{plot3}(\text{linel}(1,:), \text{linel}(2,:), \text{linel}(3,:)) \\
\% \text{hold on} \\
\% \text{end} \\
\% \% \\
\% \text{tot2} &= \text{tot} - \text{max}(\text{max}(\text{tot})); \\
\% \text{PlotZ} &= \text{vec2mat}(\text{tot}, \text{Resolution}); \\
\% \text{PlotX} &= \text{vec2mat}(\text{Int3} \text{matrix}(:,1), \text{Resolution}); \\
\% \text{PlotY} &= \text{vec2mat}(\text{Int3} \text{matrix}(:,2), \text{Resolution}); \\
\% \% \\
\% [c_{\text{2k}}, -] &= \text{Get}_\text{Zernike2}(\text{PlotX}, \text{PlotY}, \text{tot}); \\
\% [c_{\text{2k}}, -] &= \text{Get}_\text{Zernike2}(\text{PlotX}, \text{PlotY}, \text{tot} - \text{spherical.tot}); \\
\end{align*}\)

Generates the pupil coordinates and ray initial position (source) and their direction.

\begin{verbatim}
function [DIR,y,x]=RayGen_DIR(NA,n,Resolution)
    \% Generates a number (given by Resolution) of unit vectors
    \% corresponding to the direction of the outgoing rays from the Sample
\end{verbatim}
% DIR cells of units vector Dir{n}=[DIR_x;DIR_y;DIR_z]
assert(mod(Resolution/2,2)~=0,'No Chief Ray if Resolution not odd!')

phi = 0:2*pi/(Resolution-1):2*pi;
theta=-asin(NA/n):asin(NA/n)*2/(Resolution-1):asin(NA/n);

[TH,PH]=meshgrid(theta,phi);
xx=x(:);
yy=y(:);
z=cos(TH);
vec=[xx' ;yy' ;zz'
end

Intersection with a plane:

% A x + By+ Cz +D =0, plane eqt.
% N : Plane Normal [A;B;C] (3 Vector)
% LOC in : Incoming Ray Starting Point (3 Vector)
% DIR in : Incoming Ray Direction (3 Vector)
% z : Chief Ray length (-D/C) (Scalar)
% LOC out : Intersection on Plane (3 vector)

function LOC_out=PlaneLOC(N,z,LOC_in,DIR_in)
D=-N(3)*z;
t=-(dot(N,LOC_in)+D)/(dot(N,DIR_in));
LOC_out=LOC_in+t*DIR_in;
end

Intersection with a sphere and surface normal vector at the intersection:

function [ Loc_out N ] = SphereLOC( r,0_center,Dir_in,Loc_in )
A=dot(Dir_in,Dir_in);
B=2*dot(Dir_in,(Loc_in-0_center));
C=dot(Loc_in-0_center,Loc_in-0_center)-r^2;
Intersection with a Quadric and surface normal vector at the intersection: (this has not been tested on the scope, only compared with Zemax visual studio and comparison result were in a good confident intervall)

% \( F(x,y,z)=Ax^2 + By^2 + Cz^2+ Dxy + Exz + Fyz+ Gx+ HY + Ix +J=0 \)
% \( p \): initial position
% \( d \): direction
% \( c=[A \ B \ C \ D \ E \ F \ G \ H \ I \ J] \)
% \( z= f(x,y) \) is the height
function \([\text{LOC}_\text{out}, N] = \text{Quadratic}_{\text{LOC2}}(c,z,p,d)\)
t=t0;
end
t=tD;

LOC_out=p+t*d;

N(1)=2*c(1)*LOC_out(1)+c(4)*LOC_out(2)+c(5)*LOC_out(3)+c(7);
N(2)=2*c(2)*LOC_out(2)+c(4)*LOC_out(1)+c(6)*LOC_out(3)+c(8);
N(3)=2*c(3)*LOC_out(3)+c(4)*LOC_out(1)+c(6)*LOC_out(2)+c(9);
N=N/norm(N);

Quadric surface fitting: (not tested. more general surface should be included)

% Last Square, Surface Quadratic Polynomial Fitting
% vectors are in line
%p(x,y) = a1 + a2*x + a3*y +a4*x^2 + a5*x*y + a6*y^2
%E(p) = sum_0^N (p(x_i,y_i)-f_i)^2

function a = QuadraticFit(x,y,z);
N=length(x);
ein=ones(1,N);
V=[ein' x' y' x'.^2 x'.*y' y'.^2]; % the 6 Basis function for Quadratic
a=pinv(V'*V)*V'*z';
end

Cosine Formel for the new direction vector (to be calculated after each intersection)

% n_r: ratio between previous refractive index to new n(2)/n(1);
% N : surface normal
%DIR_in : Incoming Ray Direction (3 Vector)
|DIR_out : Outgoing Ray Direction (3 Vector)

function DIR_out=RefractedDIR(n_r,N,DIR_in)
DIR_out=(n_r*dot(-N,-DIR_in)-sqrt(1-n_r^2*(1-
end
Voltage to be applied on the DM: (in fact this part just saves the data in various files, the actual voltage calculation is in the function DMContorlSignalfromZerink, this part is written by T. Xiaodong and will not be included here.

1 function fataloutput = Voltage(syst,c_Zk,d,i)
2 dt=date;
3 depth_of_stage=syst.wd;
4 refr=syst.n;
5 fataloutput=cell(1);    
6 path=['Voltages_DM/' dt(1:6) '_' num2str(depth_of_stage)];
7 if ~exist(path, 'dir') mkdir(path)
8 end
9 filename = [path '/volt_' ...
10 '_WD' num2str(i) '.txt'];
11 filename2 = [path '/zernike_' ...
12 '_WD' num2str(i) '.txt'];
13 [~, force_centerV]=DMContorlSignalfromZerink(c_Zk,filename);
14 if ~isempty(force_centerV) (force_centerV >200) %Not exceed 200V on DM
15 disp(['no corr' num2str(refr)])
16 fataloutput{z}=[refr,d] ;
17 z=z+1;
18 end
19 fout2=fopen(filename2,'wt');
20 fprintf(fout2, 'wd depth: %f %f
', [syst.wd d]);
21 fprintf(fout2, 'RI %f %f %f
', refr);
22 fprintf(fout2, '%f 
', c_Zk);
23 fclose(fout2);
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