Multiwavelength confocal fiber optic microscopy

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Abstract. We present a multiwavelength fiber optic confocal microscope (MCFOM) based on the use of a wide spectral emission light source and some commercially available fiber optic components to make a reliable and compact system. We use single mode fiber optics SMF-28 to illuminate and collect the reflected light from the sample avoiding the use of a pinhole as a filter for the unfocused planes; as well as two lenses to collimate the light. The chromatic aberration produced by the lenses is used to interrogate several planes simultaneously, eliminating the confocal scanning movement obtaining an average confocal resolution of 4 μm and a surface scanning resolution of 1 μm. We demonstrated enough sensitivity to scan biological samples with a depth of penetration of 26 μm showing the potential use of this proposal in research laboratories as an alternative low cost confocal microscopy.

1. Introduction

Confocal microscopy (CM) was first described by Minsky in 1957, since then different versions have been developed to perform accurate measurements of surface profiles with millimetric or micrometric scales. A confocal microscopy system focuses light at different depths within the sample by using optics to focus source light passing through an aperture into an object and to detect light reflected or scattered from the object back to the same aperture [1]. Its basic principle lies in the ability to remove reflected light from out-of-focus planes by improving the signal-to-noise ratio for 3D image reconstruction. The CM offers several advantages over conventional optical microscopy being the most important the ability of mapping the depth of the sample. Today confocal microscopes are relatively easy to operate and have become part of basic instrumentation for image acquisition in many research laboratories and industry.

Nowadays, there is special interest in the use of the CM in applications such as the imaging of biological samples and semiconductor materials, where high resolution is required in both the surface and depth planes [2-5]. It has become a widely used commercial tool that is in a process of constant improvement. Conventional confocal microscopes perform point-by-point scanning in both planes in order to image 3D structures using a monochromatic source known in the literature as "single point" visualization. However, this configuration is relatively slow in terms of scanning speed [5]. Another confocal approaches use chromatic confocal configurations to reproduce colour of the scanned objects [6] but also include light sources with a broad spectral emission typically of hundreds of nm in which each wavelength carries information from a particular point in the sample corresponding to different levels of depth on the confocal axis, so that the spectrum of reflected light is collected, spatially filtered and finally detected by a spectrometer allowing faster 3D imaging [7]. In these multiwavelength confocal systems, a three dimensional scanning problem (x, y, z) is reduced to a 2D problem for (x, y) or (x, z) scanning. More recently, the CM technology introduced the use of fiber optics (FO) for light propagation by improving mechanical stability and integration achieving deep
scan resolutions at micrometric scales [8]. The FO confocal systems were easily introduced for *in vivo* measurements in endoscopy images including a wide variety of biomedical applications [9]. The use of FO for light illumination and collection of reflected light motivated the elimination of the aperture, typically a pinhole, by using the same FO for spatial filtration of the unfocused planes taking advantage of the small core diameter and eliminating light interference from edge imperfections of commercial pinholes due to the manufacturing process [10].

In this work, we propose a FO confocal microscope that combines: i) the principle of multiple wavelength detection for the measurement of light reflected from different focal planes formed by the chromatic aberration of a pair of lenses and ii) the use of the FO as a spatial filter to collect the light reflected from the unfocused planes as a proposal of confocal microscopy at an affordable price for research laboratories.

2. Design of a multiwavelength confocal fiber optic microscope (MCFOM)

The confocal microscope proposed in this work is shown in figure 1, it is mainly composed of the stages of: lighting, collimation, focusing, spatial filtering and detection. To explain its operation, two optical path analysis are presented: transmission and reflection. The first path begins in the illumination stage, which is constituted by a source of wide spectral emission in the visible range connected to the port B of a 2x1 fiber optic coupler. The light exits through port A and then passes to a collimation stage formed of two equal biconvex lenses (L1, L2). The L2 lens is placed at a distance of twice the focal length of L1. The chromatic aberration produced by L1 and L2 causes the path for the different wavelengths to be focused at different depth within the sample by the 60x objective of microscope. In this set up there are two key parameters: the spatial resolution (Δx) and the axial resolution (Δz).

![Figure 1. Schematic diagram for the multiwavelength confocal fiber optic microscope - MCFOM.](image)

For the path in reflection, the light focused on the sample is then reflected back through the same optical path. Passing through the objective lens, then through the collimation stage, it enters through the SMF-28 fiber to port A of the coupler where the signal will be spatially filtered. The spatial filtering is calculated by the numerical aperture of the fiber and the optical characteristics of the lens L1. Finally, once the light enters to the port A of the coupler, it pass to the port C to be detected by a spectrophotometer. The spectrum data will allow a simultaneous analysis of the reflectance signal for all the wavelengths corresponding each one to a specific confocal point. The image will be
reconstructed from the reflected intensity of the light from each scanned point within the sample. This means that each wavelength will be focused at a specific depth of the sample, so the 3D image could be reconstructed from the reflectance spectrum values taken by scanning in the xy plane with motorized micrometer stages.

We use for L1 and L2 lenses of glass BK7 with a radius of 93 mm and focal length of 100 mm. We numerically model the optical rays trace with the software Zemax to obtain the axial resolution. We made a programme in MATLAB to process all the acquired spectra obtaining the maxima for each wavelength that correspond to the different confocal planes from which we construct a matrix for the intensity profiles that allows to represent the topography of the samples.

3. Characterization of the multiwavelength confocal fiber optic microscope - MCFOM

The performance of the MCFOM involves is determined by the axial and transverse resolutions and the spatial filtering.

3.1. Axial resolution

To characterize the axial confocal resolution that represent the distance in depth corresponding to the confocal planes to be interrogated, we use a mirror, with 99% reflectance, in the place of the sample and measure the transmission spectra moving the mirror. We set the initial position of the sample using a monochromatic laser source at the wavelength $\lambda=660$ nm that provides an initial reference for the scanning the z axis. Once the maximum intensity at this point was located, a z-axis scan was performed, now using the illumination of a wide spectral emission light source (see figure 2), we sought to find the largest number of experimentally well-defined confocal planes within the range of 560-680 nm.

![Figure 2](image1.png)  ![Figure 3](image2.png)

**Figure 2.** Transmission spectrum of the wide spectral width source measured using a SMF-28 fiber.  **Figure 3.** Transmission spectrum for the confocal planes characterization.
In figure 3, we present the relative intensity spectrum obtained by moving the mirror in the z-axis over a total distance of 120 μm, we found five confocal planes where the relative intensity detected by the spectrophotometer is maximum. From these measurements, it is possible to see well defined maxima with a narrow FWHM of some nanometers (see Table 1). It is worth to mention that other wavelengths were discarded because they overlapped. In Table 1, we summarize the results for the depth distance ($\Delta z_{exp}$) for each maximum found by moving the mirror using a motorized micrometric stage in steps of 1 μm. We also compared these experimental values with those calculated with Zemax simulations ($\Delta z_{calc}$) that show a relative error of less than 10%, unless it is the wavelength of 599 nm, in which the error is higher probably due to a local defect in the optics.

| Wavelength [nm] | FWHM [nm] | $\Delta z_{exp}$ [μm] | $\Delta z_{calc}$ [μm] | Error [%] |
|-----------------|-----------|-----------------------|-----------------------|-----------|
| 583             | 6.0       | 0                     | 0                     | 0         |
| 591             | 5.0       | 4.0                   | 3.6                   | 10        |
| 599             | 6.0       | 9.0                   | 7.0                   | 22        |
| 632             | 7.0       | 18.0                  | 16.2                  | 10        |
| 665             | 4.0       | 26.0                  | 24.5                  | 6         |

3.2. Spatial resolution
To determine the experimental transverse resolution, we scanned a reflective element made of a thin layer of metallic gold in the form of a comb with fingers of 5 μm wide. We obtained a resolution of $\Delta x=0.4$ μm indicating the minimal distance the stepper motor must be moved to perform a correct scan in the xy plane.

3.3. Space filter
The optical fiber acts as spatial filter in which light coming out of the angular cone defined by its numerical aperture is disregarded. Comparing the numerical aperture of the FO ($AN_F=0.14$) and that of the lens L1 ($AN_L=0.13$), thus the beam emerging from L1 will enter the optical fiber, because its optical fiber acceptance cone is larger than that of the lens.

4. Experimental results
We did a 3D topographical reconstruction of a mosquito as a biological sample. Then, we compared the MCFOM 3D image with that obtained with a commercial Nikon A1R+ STORM confocal microscope to verify the performance of the device proposed in this work.

Figure 4 a) shows the image of the mosquito under study obtained with an optical microscope at 60x. In the inset, we point out the zone of the head of the mosquito (1 mm x 1 mm) that we scanned with both confocal microscopes. In figure 4 b) we show the 3D image obtained for an average of 10 measurements obtained with the Nikon confocal microscope with a spatial resolution of 0.1 μm, a spatial filter of 10 μm, an objective 60x and monochromatic sources in the range of 500 to 700 nm. This image reproduces well the shape and details of the mosquito that can be observed with a better contrast than the one obtained with the optical microscope corroborating the usefulness of the confocal microscopy.
We scanned the head of the mosquito, in the same 1 mm square zone, with the multiwavelength confocal microscope (figure 5 a)) by moving the motorized micrometric stages in the transversal xy plane without any movement in the z axis, once the initial position was previously determined as described in section 3.1. For comparison, we plotted in figure 5 b) the image taken with the Nikon confocal microscope but this time, with the same axial (z) and transversal (xy) resolution of the MCFOM. It can be seen that both images have a good similarity in the edge definition and most of the details, however the image formed by the MCFOM highlights less the low contrast details since only three confocal planes are measured due to the attenuation of the deeper planes.

To quantitatively compare the images in figure 5, we related its intensity using square windows composed of n x n pixels. Initially, we took a window of 4 pixels (n=2) to compare the intensity of the whole image averaged individually for each window, we finally obtain the similarity of the images in percentage. This process was carried out for each xy plane. In Table 2, we summarize the percentage of similarity in the images for different window sizes. The image with the highest similarity (62 %) is that corresponding to the first plane (z=0) analyzed with a 4 pixel window. The percentage of
similarity decreases for the deepest confocal plane probably due to light attenuation and a possible difference in the position of the confocal planes we compared.

Table 2. Percentage of similarity in the mosquito 3D image taken with the MCFOM and the Nikon CM for each confocal plane.

| Pixels (n) | Confocal plane (z=0 μm) | Confocal plane (z=4 μm) | Confocal plane (z=9 μm) |
|-----------|--------------------------|--------------------------|--------------------------|
|           | Similarity (%)           | Similarity (%)           | Similarity (%)           |
| 2         | 62.0                     | 40.5                     | 30.0                     |
| 3         | 50.6                     | 31.4                     | 19.2                     |
| 5         | 42.4                     | 22.0                     | 9.8                      |
| 10        | 32.8                     | 14.6                     | 5.4                      |

5. Summary
We present a multiwavelength confocal fiber optic microscope that uses the chromatic aberration produced by a pair of lenses to simultaneously interrogate several confocal planes each corresponding to a different wavelength thus eliminating confocal scanning motion within the sample. The proposed configuration exhibits a micrometric resolution of 4 μm with a potential depth penetration as high as 26 μm. The configuration is simplified as the fiber optic is used for light propagation and as a spatial filter eliminating the use of an additional pinhole. We demonstrated the use of this proposal to obtain confocal images of biological samples, however the axial resolution must be improved to increase the number of confocal planes, thus improving confocal resolution.

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