Three Dimension Optical Microscopy Using Structured Illumination

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ABSTRACT:
This project aims to accomplish 2D sectional scanning and 3D image generation for microstructures. The project can be applied to generate 3D image for objects at micrometer scale, and its wide application covers material science, medical science and so on. The approach to get 2D sectional images is using structured illumination, and then stick those 2D sectional images together to get the corresponding 3D images. The structured illumination projected on the microstructure in micrometer scale consist of spatial fringe patterns in a group of three with the phase interval of 90°. The spatial fringe patterns will attenuate with defocus, so that the microscope images efficiently only the portion of object where the fringe pattern is focused in. By gradually moving the sample stage, the sectioning scanning can be done for the microstructure. By processing the illumination intensity of the images illuminated by the group of successive three spatial fringe patterns, the algorithm can reconstruct the actual 2D sectional views for a microstructure. A stack of 2D sectional images can then contribute to a 3D image for the microstructure. This project is able to obtain a grayscale 3D image for the object at micrometer scale with resolution of 20 μm in x and y axis and 1μm is z axis.

INTRODUCTION:
The 3D image generation has a wide application including materials science, biomedical, medical science and so on. It can be used to observe live cell, material deflection and many microstructures. The most advantage of this technology is to add one more dimension for the observation to an object. Only by taking the traditional 2D images, this technology can provide us with the observation not only from top view, but also from side view, which enables a closed and detailed look at the observation object.

The basic methodology is first using structured illumination to get the sectional image for the objective. The constructed sectional image can get rid of blur regions which are defocused and then only observe the focused part of the target object. Then, as shown in Figure 1, by sticking those sectional images together along the third dimension, a 3D image of the observant object can finally be generated.
THEORETICAL BACKGROUND

The most important theory for this project is to use structured illumination to get the sectional image with defocused area eliminated.

By projecting a spatial fringe pattern onto an object, only the zero spatial frequency does not attenuate with defocus. As a result, the microscope images efficiently only the portion of object where the fringe pattern is focused in. [1]

By projecting pattern shown in figure 2 with 0°, 90° and 180° phases, the illuminated intensity distribution where the object is focused in will follow the sinusoid waves at corresponding phase as shown in figure 3 without attenuation.
The captured three sequential image’s intensities, which are illuminated by three arrangements of patterns with a phase-shift of $\epsilon = 90^\circ$ between two adjacent structured patterns can be processed using Eq.1, where $I, I_{0^\circ}, I_{90^\circ}, and I_{180^\circ}$ represents the intensity at each pixel for actual sectional image, photos illuminated by $0^\circ$, $90^\circ$ and $180^\circ$ phase patterns respectively. Based on this equation, the focused area without intensity attenuation will always have the highest intensity, and the defocused area’s intensity would be lower. So that the difference between focused area and defocused one can be figured out to get construct the sectional image with undesired blur illuminated.

$$I = \frac{1}{2} \sqrt{(2I_{90^\circ} - I_{0^\circ} - I_{180^\circ})^2 + (I_{180^\circ} - I_{0^\circ})^2}$$

(Eq.1)

**PROJECT CONSTRUCTION**

This project can be divided into two major parts, the first one is the hardware design and manufacturing and the second part is image processing.

**Hardware design and manufacturing:**

**Microscope control system**

The focused section adjustment can be realized by rotating the microscope’s knob to adjust the sample stage height. Then a step motor considered to control the knob, which is able to rotate the knob to a certain degree at each step. By continuously controlling rotating steps for the motor, we can adjust the height of the sample stage to get sectional scanning for the observed object at different focus.

A PKP546MN18A stepping motor from ORIENTAL MOTOR CO, LTD is adopted to adjust the knob. The motor is able to rotate 0.36 degree per step either clockwise or counterclockwise, which enables the sample stage to adjust the height for at least 1 $\mu m$ each time. The Motor control system is shown in Figure 4. The user can control the motor by input the desired rotation direction, rotation speed and step numbers to the Arduino MEGA 2560 board. Then the Arduino Board would transmit the order into the corresponding control signals for the motor to the motor driver. And the motor driver is responsible for power supply and controlling the motor.
The centers of motor and knob are required to match to prevent the eccentric phenomenon. However, there is a big difference between the heights of motor shaft center and knob center. So I designed a stage to support the motor, which is also able to adjust the location of motor. The design is presented in Figure 5. The slots on the motor stage and the L shape support stand for motor provide a large room for the adjustment of motor location. A shell is also designed to prevent the outside disturbance on knob during the measurements as shown in Figure 5.

**Projector setup**

A projector is used to project structured illumination on the object. There is still a high difference between the heights of projector and microscope inlet. A stage design for the projector is shown in Figure 9. A shell is designed to fit the modified projector as shown in Figure 10 and a connector is also designed to prevent the light pollution from outside when projecting the images to the microscope shown in Figure 11.
Projector selection and modification

The projector should be able to provide high contrast images because the structured illumination only consists of black and white patterns. The difference between black and white patterns should be obvious to provide a high-quality structured illumination.

The projector should also provide high resolution. Given the same microscope magnification ratio, higher resolution can provide more structured patterns projected on the observation object. So that a higher resolution observation can be made, thus a more detailed and accurate 3D image can be generated.

A projector with 1280*800 resolution and 1500:1 contrast ratio is selected.

However, the original smallest projected image has a size of 64(cm)*16(cm), then only a group of white and black patterns can projected on the observation object, then the structured illumination does not work. Additional convex lens is need to scale down the projected image.

A convex lens with focus of 25.4mm is adopted to scale down the smallest clear image to a size of 96mm*16mm, which provides around 80 groups of white and black patterns on the observation object. The modified projector is shown in figure 12.
Microscope fix

According to the basement shape of the microscope, I designed two protector for the microscope which can be mounted on the stage to prevent the accidental moving of microscope during the measurement as shown in Figure 13 and 14.

Manufacturing

All the parts are manufactured using a 3D printer. The 3D printer would automatically generate build path and then print it according to the designs drew by Solidworks 2015.

Working procedure

The integrated system is shown in Figure 15. The projector would first project structured illumination to the microscope. At the same time, the step motor is on to rotate the knob then to adjust the height of sample stage. At each height, the projector would project three different structured illumination on the observation object and the camera would take photo for each
illumination on the object. And then those images would be processed to finally get a 3D image for that object.

**Figure 15.** Integrated 3D image generation system

**IMAGE PROCESSING:**

A tissue fiber with diameter around 50 μm was observed for illustration. A 40X objective lens is used for the microscope. The AMSCOPE software is used to record and snap the structured illuminated image of the object. Figure 16, 17 and 18 shows the images for a tissue fiber at the same height illuminated by 0°, 90° and 180° phase patterns respectively.

**Figure 16.** Tissue Fiber projected by 0 phase fringe patterns.  
**Figure 17.** Tissue Fiber projected by 0 phase fringe patterns.  
**Figure 18.** Tissue Fiber projected by 0 phase fringe patterns.
MATLAB R2015a Research version is used to process the data. The photos are first read by MATLAB. The original color images are transferred to grayscale using the MATLAB function “rgb2gray” to analyze its intensity. Based on Eq.1, the intensity at each pixel was reconstructed and a processed 2D sectional image for the tissue fiber is shown in Figure 19.

![Figure 19. Original constructed 2D sectional image for a fiber tissue](image1)

According to Eq.1, regions within focus would have the higher intensity, which should be bright on the photo and region without focus should have lower intensity as dark parts shown in the picture. The rod like shaded area was the defocused section of fiber and the bright spots on that should be the area on focus at that height. The focused and brightest points are important to figure out the shape feature of the tissue fiber at that section. So the intensity is first normalized from 0 to 255 to increase the contrast, and make it easier for the selection of focused points. The results were shown in Figure 20.

![Figure 20. Intensity normalized sectional image for a fiber tissue](image2)

The circled area indicated the sections seem to be on focus. To make it clearer, the scaled intensity distribution of this photo is analyzed, and 98 percent of the total pixels’ intensities are found to be lower than 200. The focused area should have the highest intensity, so a high pass filter with a 200/255 band is adopted to block the defocused points which provides a much clearer look at the focused feature on the picture as shown in Figure 21.
For a fiber tissue, whose shape is like a cylinder, the sectional view at each slide should have a counter profile as the red loop indicated in Figure 22. The most important feature at each slide when integrating a stack of 2D sectional images to form a 3D one is the outmost feather at each slides. Considering the present noise of the photo, the counter profile needs to be fit at each slide to make a continuous shape feature.

Eighth Fourier series are used to fit the curve. However, it’s hard to fit the closed loop in Cartesian coordinate system. For example, if the origin is set at the lower left point on Figure 22, the Fourier series would merge the closed loop. And the location selection is different for different contour profiles at different slides. A polar coordinate is better for the analysis of a closed loop. First, the center of the closed loop is found and set as the origin for a polar system, and the location information relative to the origin points are transmitted into the polar system. The eighth order Fourier series method according to Eq.2 is used make the best fit of the counter profile.

\[
\rho = a_0 + \sum_{n=1}^{8} a_n \times \sin(n \times \theta \times w) + \sum_{n=1}^{8} b_n \times \cos(n \times \theta \times w)
\]  \hspace{1cm} (2)

An example of the 8th Fourier series at slide 35 is shown in Figure 23, which give an R-square value of 0.8648 indicating the Fourier series is able to provide a good fit for the curve.
The fitted curve is transferred back to Cartesian coordinate system because the center points at different slides were at different location, so it difficult to directly integrate the curves for all the slides in polar coordinates to generate a 3D view. The focused points at corresponding heights are plotted, and then the 3D image for a fiber tissue is shown up in figure.21. The color difference indicated the difference in height. The color from dark to light represents the corresponding height from low to high. The outmost feature of the tissue fiber at each slide can be figured by the shape of plot at each height. The dimension for this 3D image is $51\mu m \times 2000\mu m \times 6000\mu m$. 

Figure 23. The eighth Fourier series fit for the contour profile on slide 35 in polar coordinate

Figure 24. 3D image for a fiber tissue
**IMPROVEMENT:**

There is a large room left for improvement for this project in terms of image resolution, noise filtration and processing speed.

**Image resolution:**

Present resolution is limited to $20\mu m$ at each slides, which is determined by the width of fringe patterns projected on the microstructure. However, only around $1/10$ of the total stripes out from the projector are used. The resolution can be increased by 10 times if all the projected structured patterns can be used.

Under the condition that the microscope magnification rate is fixed, the goal is to scale down the input image of the microscope.

The first possible solution is to change the projector original lens. Since the projector is originally used to magnify the image, so the original lens must concave lens. The concave lens can be replaced by convex lens to scale down the projected image to increase resolution.

Another possible solution is to design a convex lens combination to scale down the projected image without changing the original lens. But the input image intensity would be a problem for this solution, because there would be more light loss than the first possible solution. The light intensity is critical to illuminate the project especially half of the projected patterns are totally dark.

**Noise filtration:**

Two steps have been already used to filter the noise. The first one is to scale up the intensity to increase the contrast and increase the intensity difference between the focused area and defocused area. The second step is to adopt a high-pass filter to pick up the points with the highest intensity. However, there were still many noises outside the shape of fiber tissue as shown in Figure 21, which would definitely influence the selection of focused points and decrease the 3D image quality.

Plus, the noise filtration mainly depends on the data processing for now, which could cause distortion or loss of the actual data. And the final 3D image may not show the exact feature of the microstructure observed. The source of those noises need to be found out and eliminated.

A photo using the same sectional image construction strategy is made directly for three structured illuminations without any observation objects. Theoretically, the constructed photo should be totally bright because nothing is out of focus. However, the actual photo is shown by Figure 25, from which the fringe patterns can be clear figured, which are the noises for 2D sectional image scanning. This phenomenon indicates some projection problems.
The first guess is that there exists interference for the reflected light, thus causing those patterns. The possible solution is to use some low coherence light source for the projector to eliminate the interference influence.

The second guess is about the projected patterns. Three arrangements of white and black patterns as shown in Figure 2 are used to simulate the sinusoid intensity distribution. However, since only four patterns are used for now for each arrangement, the actual projected intensity may not fit the sinusoid wave that well, which will also cause the existence of those patterns based on Eq.1. The possible solution is to use more patterns with different gradually changed gray scale ranging from 0 to 255 to simulate the sinusoid intensity distribution. A better simulation for the sinusoid intensity distribution would fit Eq.1 better, and gives more accurate constructed intensity.

**Data processing:**

For now, the data processing speed is not satisfying. It takes around one second to snap one picture from the camera and it takes around 10 minutes to generate a 3D images for only 51 slides.

The possible solution to reduce the photo taking time is to record a video and then process it directly. And 3D generation algorithm can be optimized. The improvement of projection quality as disused above for the noise filtration would also cut some time-consuming steps for image processing.

**REFERENCE**