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DESIGN AND FUNCTION OF A STRUCUTRED ILLUMINATION SYSTEM FOR USE IN AN AO MICROSCOPE

A thesis submitted in partial satisfaction
of the requirements for the degree of

MASTER OF SCIENCE

in

ELECTRICAL ENGINEERING

by

Matthew Kissel

September 2014

The Thesis of Matthew Kissel
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Vice Provost and Dean of Graduate Studies

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

**DESIGN AND FUNCTION OF A STRUCUTRED ILLUMINATION
SYSTEM FOR USE IN AN AO MICROSCOPE**

Matthew J Kissel

The design and function of a Strucutred Illumination system for use as part of an Adaptive Optics Structured Illumination Microscope (AOSIM) is presented. After successful implementation of AO in a standard widefield microscope, our lab wanted to apply the technology to a more advanced imaging modality that has a lot to gain from the AO system. SIM allows us to reconstruct an image with resolution beyond the Rayleigh limit of the optics, called a super-resolution image, by aliasing high spatial frequencies, outside the limit of the optics, to lower frequencies within the system pass band. The aliasing is accomplished by spatially modulating the illumination at a frequency near the cutoff frequency of the system. These aliased frequencies are superimposed on the lower spatial frequencies of the object in our image. Using multiple images and an inverse algorithm, we separate the aliased and normal frequencies, restore them to their original frequency positions, and recreate the original spectrum of the object. This allows us to recreate a super-resolution image of the object. We show that our SIM system creates images that are a higher resolution than a standard wide-field microscope.

1. INTRODUCTION

Structured Illumination Microscopy is a super-resolution imaging modalityof interest to the cell biology community due to it's ability to resolve features up to half the size of a conventional wide-field microscope with fairly minimal additional equipment and costs. However, the quality of reconstruction is affected very heavily by aberrations created by the sample being imaged, making it a very difficult method to use in-vivo. This issue of aberration in in-vivo imaging has been explored by our lab before and alleviated through the design of Adaptive Optics systems in wide-field, confocal, and 2-photon microscopes. Developing a SIM system to do AO microscopy research on is a natural extension of these previous exploits in the field. We selected SIM due to this inherent requirement that the source images use be diffraction limited or at least fairly free of aberration to be effective. Also, SIM is a wide-field technique and as such can be easily and cheaply built into our existing system.

One important science case that exemplifies this need for super-resolution imaging in-vivo is the study of chromosome structure and function during meiosis. One major barrier to imaging chromosome structure during meiosis the is the fact that the early stages of this process will typically occur towards the middle of the embryo, and thus imaging these early stages of meiosis requires focusing through the embryo material, which causes aberrations. For effective imaging of this process, the system requires the ability to perform dynamic in-vivo imaging

through thick tissue (10-100 μm) with 1 Hz frame rates and 100 nm resolution. This level of super-resolution has also enabled imaging of the chromosome structure in *C. elegans* for limited depth below the coverslip (16 μm), where the depth was limited by the sample-induced aberrations, which should be correctable by AO.[4] The development of a SIM system to function with our existing AO wide-field scope will allow these requirements to be met.

2. THEORY AND BACKGROUND

2.1 The Diffraction Limit of Microscopes

The spatial resolution of an optical imaging system can be described as the smallest distance at which two radiating points will be visually distinguishable as separate. For example, on a system with 500nm resolution, two point sources 500nm apart will just be distinguishable as two separate points, but if the point sources are 250nm apart, they will appear as a single point. When designing an imaging system, an optical engineer generally makes their selection of lenses, lens arrangement, illumination source, etc. in a way that will reduce optical aberration, and thus maxmize the system resolution. If designed carefully and properly aligned, the resolution of the system will be limited not by the design, but by physical limit created by the diffraction of light, called the Diffraction Limit. In this case, the resolution limit depends only on the wavelength of the illumination source and the Numerical aperture of the objective, according to the relation: $f_o = 2 * NA / \lambda$. Note that instead of representing the resolution as a size, we represent it as a spatial frequency. This is convenient because we can consider our imaging system as a spatial lowpass filter, where f is the cutoff frequency of the filter. Also, the SIM technique makes extensive use of frequency domain processing, so it is useful to think in terms of frequency.

2.2 Structured Illlumination

Structured illumination microscopy can increase the resolution of a wide-field microscope, by bringing spatial frequencies that are above the cutoff frequency

down into the pass band of the lens. This is accomplished by utilizing a spatially modulated source, with a frequency close to the cutoff frequency, to illuminate the object in focus. The spatial modulation aliases the frequencies above the modulation frequency into the pass band of the system, overlapping the frequencies that are naturally there. Using Fourier domain processing, we can algorithmically separate the normal frequencies from the aliased and overlaid frequencies using multiple images, with multiple parameters of the modulation. We then move the aliased frequencies back to their original position and reconstruct an image of the object with the higher frequency detail than the system alone could image, increasing its resolution.

Structured illumination can also provide axial sectioning. The OTF of a system with defocus has much lower gain at the high spatial frequencies than a system with no defocus. Objects that are out of focus will therefore have less high spatial frequency information than those in focus. Additionally the modulation illumination will be weaker as well. Since the reconstruction algorithm relies on the structure of the illumination to alias the higher spatial frequencies and bring them into the pass band of the system. It effectively selects the high frequencies of those objects that are in the focal plane to reconstruct and thus performs an axial sectioning of those images.

This method can achieve the highest resolution when the optical system also has the highest resolution. However, if there are aberrations in the light path caused

by the sample or the system, the structure of the illumination will be degraded. This reduces both the amount of detail in the structured illumination that can be projected into the object and the detail that can be passed through it system. These kinds of aberrations increase as we try to view deeper into the tissue. If the aberrations in the optical path are too large, the illumination pattern imaged onto the object at the focal plane degrades and there is less ability to reject the out of focus light or alias higher spatial frequencies. Consequently, our ability to reconstruct the supper resolution image also degrades. [5][6]

In fluorescent microscopy for biological imaging, structured illumination has sparked a new life by providing an inexpensive, high-resolution technique for acquiring wide-field images. But, deep tissue imaging is still a major limitation for structured illumination microscopy. The tissue in the optical path introduces aberrations that degrade the imaging capabilities. This problem becomes more pronounced as the depth of the object of interest increases since more aberrations are introduced in the optical path, particularly sphere, which similarly to defocus reduces the gain at high spatial frequencies. This problem can be overcome by correcting the aberrations in the optical path. Adaptive optics has proven to be a very useful tool in overcoming this limitation, not just in wide-field microscopy but also in other high-resolution imaging techniques like confocal and multi-photon microscopy[7].

$$I_{ill}(x,y) = H(x,y) * I_{sfp}(x,y) \tag{1}$$

5

Where $I_{ill}(x,y)$ is the image of the structured illumination at the object plane, $H(x,y)$ is the intensity point spread function of the imaging system, $I_{sfp}(x,y)$ is the intensity of the single frequency illumination pattern, and * is the convolution functioin:

$$I_{sfp}(x,y)=1+m\cos\left(k_g x+\theta\right)$$
(2)

Where m is the modulation depth, k_g is the illumination pattern normalized spatial frequency, and θ is an arbitrary phase for the grating. In the Fourier domain the image produced at the object plane is given by:

$$F_{ill}\left(f_x,f_y\right)=\hat{H}\left(f_x,f_y\right)F_{sfp}\left(f_x,f_y\right)$$
(3)

Where $F_{ill}(f_x,f_y)$ and $F_{sfp}(f_x,f_y)$ are the Fourier transforms of the structured illumination and the intensity pattern respectively, and $\hat{H}$ (f_x,f_y) is the optical transfer function of the imaging system.

To produce an optical sectioned image of the object of interest, three different images are collected with three different phases (I_1, I_2, I_3 when θ =0, $2\pi/2$, $4\pi/3$). Each image contains information pertaining to the object and structure illumination as follows[12]:

$$I_n(x,y)=I_{ill}(x,y)O(x,y)*H(x,y)$$
(4)

Where $O(x,y)$ is the object of interest, and $I_n(x,y)$ is the structured illumination image (n=1,2,3) obtained for each phase (θ =0, $2\pi/2$, $4\pi/3$). The sectioned image can then be mathematically produced by using the following equation on each pixel of the previously collected images I_1, I_2, I_3: d

$$I_s = \sqrt{\frac{\left(I_1 - I_2\right)^2 + \left(I_1 - I_3\right)^2 + \left(I_2 - I_3\right)^2}{2}} \qquad (5)$$

Where I_s is the structured illumination sectioned image.[8]

2.3 The application of AO to the SIM design

As can be evidenced by equations 1-4, the success of the SIM reconstruction are heavily influenced by the PSF of the optical system and sample. While the optical system itself can be designed to be diffraction limited, the wavefront distortions introduced by the imaging through deep tissue samples are dynamic and depend on the characteristics of the tissue used, imaging depth and NA of the objective. In general, the deeper the imaging depth, the stronger the optical aberrations and the more the PSF is spread out and distorted. Thus in order to successfully reconstruct a super-resolution image beneath the sample surface, we need to use Adaptive Optics to correct for the aberrations and shrink our PSF back to a compact, diffraction-limited point. In particular, we expect that our deep tissue PSF will be distorted by a very strong amount of spherical aberration, which our selection of AO hardware, described below, has been optimized to remove.

3. HARDWARE SETUP
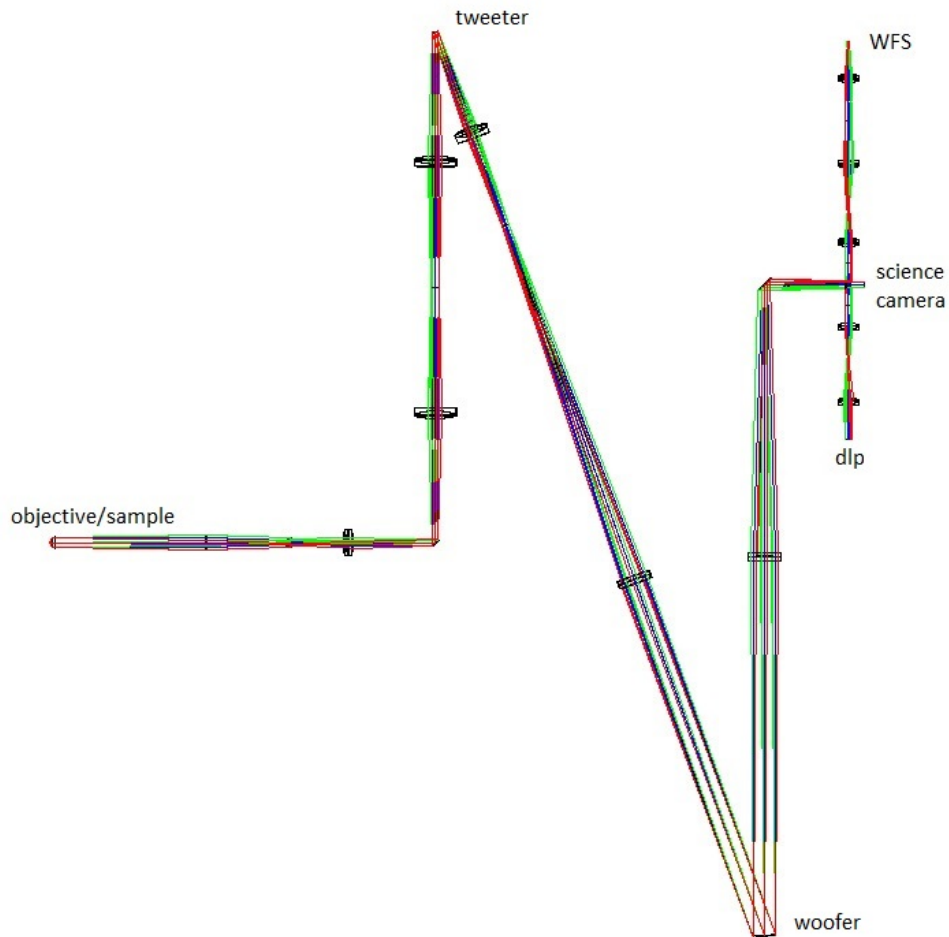

figure 1: Optical layout of our AOSIM test bench

3.1 Microscope/sample section

To simplify the design and prove the adaptability of the AO-SIM, the set-up will use an Olympus IX 71 inverted microscope frame. For setup and alignment of our SIM system, a mirror can be placed on the sample area of the scope in order to

allow us to directly view the structure of our illumination source. For actual imaging, this mirror can be replaced with fluorescent samples. Our sample at the current stage of testing consists of GFP microdots embedded in ~100 microns of agar on a standard microscope slide. In the future, this system will be adapted to fully test the AO system capabilities by putting the microdots underneath Drosophila embryos, which will introduce significant aberrations and scattering on the way to and from the microdots.

3.2 Illumination and DLP leg

Our illumination leg consists of a 60mW 488nm cyan laser. This wavelength was chosen due to its suitability for exciting the GFP microdots. In order to reduce fringe effects and speckle, the laser travels through a spinning disk spatial de-correlator that will greatly reduce the coherence of the light. The resulting beam then hits a (608 x 684 PIXEL) TI DLP, which is part of the Lightcrafter evaluation module. We use the DLP to create a binary structured illumination image in our laser light.
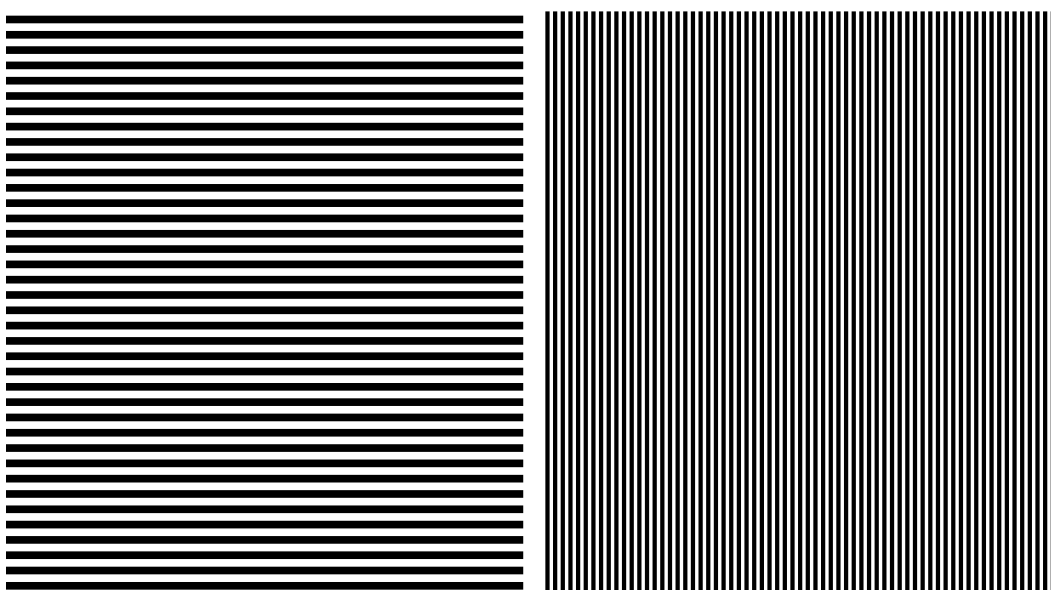
figure 2: Vertical and Horizontal SIM mask examples

The masks were created in matlab. Two example masks are shown in figure 2. The ideal frequency of the masks is determined by loading a mask into the DLP and using the mirror to view the structure. The frequency is then adapted to be very near, but not beyond the resolution limit of the system. This ensures that the resolution increase is as large as possible. While the ideal illumination pattern is a continuous sine wave, the extra high frequency components of our binary structure pattern is filtered by the system optics so that only the primary frequency is left. This blurring of the square bar pattern into sin waves tells us visually that we are near the diffraction limit of the scope.

In addition to creating the structure in the illumination source, the DLP also acts as the master timer, triggering the science camera and thus keeping the system in

sync. This is done via a hardware trigger pulse. The lightcrafter trigger module by default did not output enough current to trigger the Andor camera reliably, so an amplifier was built to ensure smooth triggering.

3.3 Wavefront Sensing and Correction

The wavefront correction leg of our optical system consists of two deformable mirrors, a woofer and a tweeter, both positioned conjugate to each the system pupil. The woofer is a 52 actuator Imagine Optics MirAO 52e magnetic actuator mirror. It was selected due to its high stroke (40 microns) and thus its suitability to correct low order aberrations, such as the strong spherical aberration typically produced by thick biological samples. The tweeter is a 140 element Boston Micromachines MEMS DM. It was chosen for its high actuator count, which we will use to correct the residual high order aberrations left after removing the low order aberrations with the woofer.

To take full advantage of the adaptive optics system compensation the proposed optical design of the system corrects both the structured illumination light on its way to the object plane and the light that it gathers from the object plane for imaging.

The wavefront data used for controlling this mirror will come from a Shack-Hartmann wavefront sensor, consisting of a 12x12 lenslet array projected onto a Princeton Instruments PhotonMAX camera. This approach was chosen over an

iterative image-optimization technique due to its faster speed, allowing our system

to image in real time.

4. SOFTWARE

4.1 Acquisition Software

We are using software bundles provided by the manufacturers of our camera and DLP to perform the acquisition procedure. For the DLP this is the lightcrafter GUI and Andor Solis for the camera. The lightcrafter GUI contains all the facilities required to upload image sequences (SIM masks) to the DLP and control the rate at which they are cycled through. Since the camera software does not directly speak to the DLP software, the use of hardware triggers was required, and is detailed in the previous section of this paper. Getting these hardware triggers to function requires enabling the DLP trigger output in the DLP software, then using the Andor software to set the cameras trigger mode to external. This way, every time a new SIM mask is loaded up, a trigger pulse is sent to the camera, telling it to acquire a new image.

Since the specimen being imaged (microdots in our case) may drift in position from image to image, we must be able to capture the entire image set before the particles have time to move. If this is not done correctly, the drift between frames will actually cause the reconstructed image to have worse resolution than the originals instead of better. To prevent this drift, we set our DLP to cycle through the mask images very quickly upon activation (about 1s per image set), and set the camera to acquire in kinetic series mode, which allows it to capture on the first n triggers and save the results to a single easy to use .dat file.

13

When our software is set up in this way, the result is a system where we can click once to immediately perform the entire image capturing process. This is necessary for the scope to be useful as a research device.

4.2 Reconstruction software

We chose to use Matlab to reconstruct the SIM images due to its ease of prototyping and our existing familiarity with the software. Our matlab code take the input .dat file containing the SIM series and performs the entire reconstruction process based on parameters set in the program. In addition, it allows to iterate parameters and perform a reconstruction for each parameter value, allowing us to easily find the correct value. The various steps in this reconstruction process are detailed below

The effectiveness of the reconstruction is heavily affected by the image noise. As such, care must be taken to remove background noise without removing any real data. We acquired all of our images using a method that effectively allowed us to threshold the image in our matlab program before applying the algorithm. First we set an integration time and gain that puts the desired signal near saturation without actually saturating the camera. This ensures that the image noise is far less intense than our signal, and can thus be separated out more easily. Next we obtain a background noise image, in which a series of images are taken with no

14

illumination at the desired gain and integration time, then averaged together. This background is then subtracted from any images taken afterward. The resulting image has a very strong signal and low residual background noise, making it easy to remove the remaining noise via thresholding without removing any actual image data.

After the images are prepared with all noise removed, the matrix operations required to reconstruct the original image are performed. In addition to performing these matrix operations, the program serves to determine appropriate initial conditions and parameters for each new data set. This is done by selecting a parameter to be iterated throughout a certain range of values, and performing a reconstruction at each of those values. The results are then viewed and analyzed to determine which setting for that parameter is correct. Some of these parameters are variables in the reconstruction equations above such as the SIM shift frequency and initial phases, but there are also other parameters that are necessary in order to apply the algorithm to pictures obtained in real optical systems, such as 'skew' (the unintentional offset angle of the illumination pattern) and the cutoff frequency of the filters used to remove high frequency noise from our images before and after reconstruction. Theoretically, in an implementation with a fully fixed/static optical system and illumination pattern, these parameters will only need to be determined once, but in any real practical implementation, some small shifting will likely occur over time, and thus this calibration can likely be

performed once per imaging session rather than once per image set as in our current system.

We have found that a good procedure is to start by iterating over the skew to select the angle that reduces image artifacts. Next, the SIM frequency is iterated over, although a very good initial estimate can be given by observing the fft of the pattern sent to a reflective target rather than the fluorescent dots. The best SIM frequency will show the most compact dots upon reconstruction. We also must iterate over both the x and y initial phases, and select the settings that lead to the least shift in the resulting image. Some inconsistency over the shift amount across the reconstructed image can point out if more tuning is required to select the appropriate SIM frequency. The final parameter that is iterated over is the cutoff of the filter used on each individual image before reconstruction, and the filter used on the disentangled/shifted images. Ideally, this should match the cutoff of our system optics pretty well. As such, we can get a pretty good estimate of this by created a series of points on an image, and increasing the cutoff of the filter applied to it until the resulting dot size is similar to that of the smallest point features being viewed by the microscope. Through iteration we can fine tune this number, as too low a cutoff will result in image artifacts, and two high a cutoff will result in image noise and artifacts
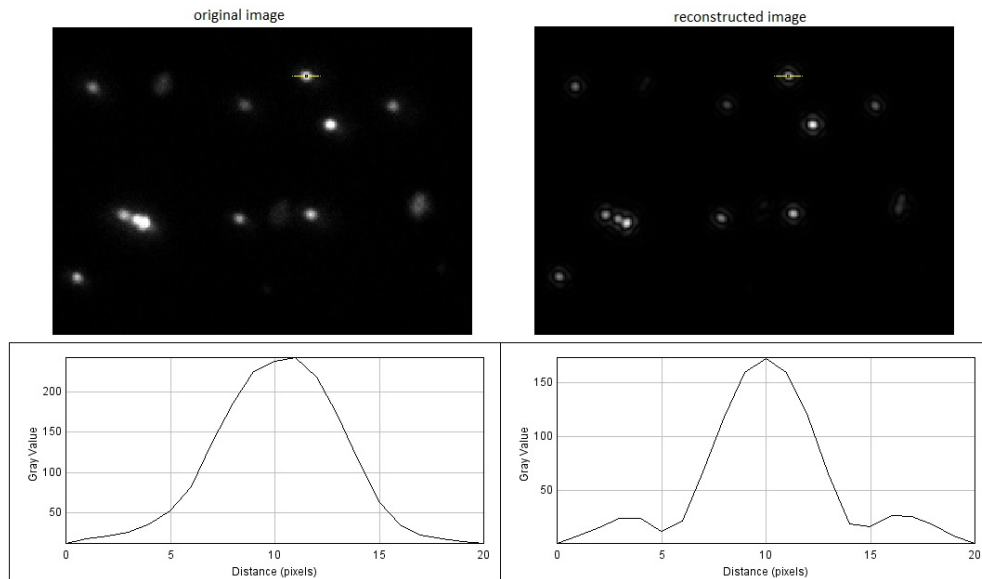
5. RECONSTRUCTION RESULTS

Figure 3. Comparison of original and reconstructed images

An example of the reconstruction algorithm after all parameters have been selected can be seen in figure 2. A few effects of the reconstruction can be seen. First, all in focus dots have been decreased in size and have more brightness concentrated in the core. This can be seen especially well in the dots that were close enough to not display clear separation on the original image. In the reconstructed image, these dots are smaller and show a clear decrease in intensity between them, distinguishing them as separate dots, as can be seen by the line profile. Another prominent effect of the reconstruction is the rejection of out of focus signal. On the original image, these out of focus signals are the dots that are much wider and dimmer. On the reconstruction, these out of focus dots either decrease in intensity or disappear altogether.

17

The rejection of the out of focus dots also exemplifies the need for AO in order to effectively reconstruct through aberrating tissue. Other distortions in the patterned illumination's wavefront, such as sphere and coma, will similarly distort the image PSF, causing some of the aberrated signal to be rejected by the algorithm.

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