High resolution, molecular-specific, reflectance imaging in optically dense tissue phantoms with structured-illumination

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Abstract: Structured-illumination microscopy delivers confocal-imaging capabilities and may be used for optical sectioning in bio-imaging applications. However, previous structured-illumination implementations are not capable of imaging molecular changes within highly scattering, biological samples in reflectance mode. Here, we present two advances which enable successful structured illumination reflectance microscopy to image molecular changes in epithelial tissue phantoms. First, we present the sine approximation algorithm to improve the ability to reconstruct the in-focus plane when the out-of-focus light is much greater in magnitude. We characterize the dependencies of this algorithm on phase step error, random noise and backscattered out-of-focus contributions. Second, we utilize a molecular-specific reflectance contrast agent based on gold nanoparticles to label disease-related biomarkers and increase the signal and signal-to-noise ratio (SNR) in structured illumination microscopy of biological tissue. Imaging results for multi-layer epithelial cell phantoms with optical properties characteristic of normal and cancerous tissue labeled with nanoparticles targeted against the epidermal growth factor receptor (EGFR) are presented. Structured illumination images reconstructed with the sine approximation algorithm compare favorably to those obtained with a standard confocal microscope; this new technique can be implemented in simple and small imaging platforms for future clinical studies.

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References and links

3. ApoTome structured-illumination head by Zeiss Inc. at www.zeiss.com
1. Introduction

Confocal scanning optical microscopy has emerged as a technique which exhibits several advantages over conventional optical microscopy. The important feature of confocal microscopy is the fact that out-of-focus blur is absent in confocal images, yielding the capability for non-invasive optical sectioning of both in vivo and in vitro specimens. This leads to the possibility of generating (3D) images of thick transparent objects such as biological cells and tissues. The simple and inexpensive alternative to confocal microscopy is the structured-illumination technique proposed by Neil, et al. This method uses the principle that all but the zero (a.k.a. DC) spatial frequencies are attenuated with defocus. This observation provides the basis to obtain optically sectioned images from a conventional wide-field microscope. A modified illumination system of the microscope projects a single spatial-frequency grid pattern onto the object. The microscope then images faithfully only that portion of the object where the grid pattern is in focus. The structured-illumination technique requires acquisition of at least three images to remove illumination structure and reconstruct an image of the layer. The schematic layout of the typical structured illumination microscopic system is presented in Fig. 1. The technique has been already applied to small pollen grains in reflectance mode and cell monolayers in fluorescence mode. Recently the technique was implemented commercially by ZEISS in the ApoTome and Thales Optem in OptiGrid heads for inverted microscopes and fluorescent applications. Additionally the system was commercialized for industrial applications (MEMS testing) by Thales Optem.

Recently, confocal microscopy has shown promise to image the morphologic features of precancerous lesions in epithelial tissue. The simplicity of the structured illumination technique provides a convenient method to obtain this useful diagnostic information in vivo. There is however no example of successful reflectance structured illumination imaging of dense tissue samples. The difficulty of reflectance imaging arises from the very strong out-of-focus contributions compared to the optical-section signal. The useful in-focus information is limited to even as low as two gray levels of the camera, and standard algorithms are not capable of effectively reconstructing images of the in-focus section under these conditions. Furthermore, reflectance imaging of tissue is generally sensitive only to non-specific differences in refractive index, and provides rather limited information about the range of molecular changes associated with pathophysiology. Therefore, to overcome these problems
we undertook two actions: (1) we applied the sine approximation algorithm for image reconstruction and (2) we used targeted reflectance contrast agents to provide a molecular-specific source of increased signal from cells overexpressing cancer-related biomarkers.

![Diagram of the typical structured illumination microscope system.](image)

**Fig. 1.** Layout of the typical structured illumination microscope system.

The sine approximation algorithm applied here in the context of structured-illumination has been previously used to monitor vibrations and to measure shape; however, in the context of image processing, this is a new approach. We find the algorithm to be very robust and, most importantly, that the sine approximation algorithm works correctly for significantly smaller dynamic range than is required by conventional structured-illumination imaging techniques. Using the sine approximation, the optical section image can be reconstructed even if the portion of the signal from the optical-section signal in each raw image is so low that it only spans two gray levels. Under these conditions, we find that the reconstructed image has similar dynamic range to that which is obtained by directly imaging the signal from the optical section.

In unstained tissue, spatial fluctuations in refractive index provide a source of backscattering which can be imaged in reflectance mode. The magnitude of the refractive index difference between the nucleus and cytoplasm in epithelial cells is small (Δn~0.02), thus the signal from the optical section is small compared to the multiply-scattered out-of-focus light. Simple, non-specific contrast agents such as acetic acid (vinegar) are used routinely in clinical cancer detection and can enhance nuclear backscattering several fold, but the magnitude of the in-focus signal compared to the out-of-focus signal is still small. Optically active contrast agents, such as gold nanoparticles, are stable, inert and biocompatible; they can provide a bright source of reflectance when targeted against disease-related biomarkers in biological tissues. In this paper, we used antibodies against the epidermal growth factor receptor (EGFR), a receptor commonly overexpressed in cancerous and precancerous tissue, to target our contrast agents. We created multi-layer epithelial cell phantoms with cells expressing levels of EGFR typical for normal and neoplastic tissue. Using this approach, we were able to distinguish images from tissue phantoms representative of normal and precancerous tissue, and obtain high-resolution reconstructed images at increasing depths. Optically sectioned images with the structured-illumination mode compare favorably to that of the “gold standard” of confocal microscopy. We found that the maximum imaging depth was limited to 50 microns beneath the phantom surface in both confocal microscopy and structured illumination; this limitation is caused by the high scattering associated with the contrast agents. We are currently studying the relation between nanoparticle concentration...
and imaging depth to optimize this procedure for imaging throughout the typical 200-300 micron thick epithelium.

To the best of our knowledge we are the first group to successfully apply the structured-illumination technique in reflectance-mode imaging of a thick biological specimen.

2. Sine approximation principle

The algorithm used for optical-section image reconstruction is based on the sine approximation method. The structured-illumination pattern, which is in the form of a sinusoidal amplitude grating, is projected on to the image plane to be reconstructed. Light remitted from the total sample is imaged onto an array of photodetectors. Acquiring structured-illumination images at different locations of the grating enables amplitude reconstruction for a given pixel in the image. The change of the grating position can be obtained with continuous grating oscillation (for example in resonance) or by stepping the grating in discrete positions. Without loss of generality, the signal at a pixel on the CCD or CMOS image sensor may be described by

\[ S(i, j, n) = A(i, j) + B(i, j) \left[ 0.5 + 0.5 \cos \left( \varphi(i, j) + n \Delta \varphi \right) \right], \]

where \( B(i, j) \) is an optical-section signal amplitude, and \( A(i, j) \) is the fluorescence or reflected background component that is independent of modulation. In practice, due to finite dimensions of a pixel, the signal \( S \) is defined for discrete locations \( (i, j) \) on the image sensor, i.e., \( i \) and \( j \) denote a row and a column in the array. The parameter \( \Delta \varphi \) is a phase interval between successive raw-image frames, indexed by \( n \). Finally, \( \varphi(i, j) \) is an unimportant absolute phase of the structured-illumination pattern with respect to the \( (i, j) \)th pixel.

From this point forward, we consider only a single image pixel. The signal at each pixel is independent and we can ignore the pixel’s particular coordinates without loss of generality. The signal observed in the \( n \)th frame is

\[ S \left( n \right) = A + 0.5B + 0.5B \cos \varphi_0 \cos \left( n \Delta \varphi \right) - 0.5B \sin \varphi_0 \sin \left( n \Delta \varphi \right). \]

The signal amplitude \( B \) and the phase \( \varphi_0 \) may be calculated as the solution of a set of \( N \) independent linear equations. The number of equations \( N \) is determined by the number of raw-image frames acquired. To clarify how the amplitude and phase are recovered, Eq. (2) is rewritten in the form

\[ S \left( n \right) = b_0 + b_1 x_1 \left( n \right) + b_2 x_2 \left( n \right), \]

where \( b_0 = A + 0.5B \), \( b_1 = B \cos \varphi_0 \), and \( b_2 = B \sin \varphi_0 \) are all time-independent variables, and \( x_1 = 0.5 \cos \left( n \Delta \varphi \right) \) and \( x_2 = 0.5 \sin \left( n \Delta \varphi \right) \) depend on time through the raw-image frame index \( n \). When \( N \) raw images are acquired, the ensemble of recorded signals at each pixel may be expressed more succinctly in matrix-vector form

\[ S = Xb + m, \]

where \( S \) is an \( N \times 1 \) vector of all \( N \) signals recorded at the pixel, \( X \) is an \( N \times 3 \) matrix filled with the predetermined values of \( x_1 \) and \( x_2 \), \( b \) is the vector \( \left[ b_0 \ b_1 \ b_2 \right]^T \), and \( m \) is an \( N \times 1 \) vector representing additive noise.

The least-squares estimate of the unknown vector \( b \) is obtained from
\[ \mathbf{b} = (\mathbf{X}^T \mathbf{X})^{-1} \mathbf{X}^T \mathbf{S}. \]  

(5)

It should be noted that the least-squares estimate returns a vector \( \mathbf{b} \) with minimum errors for any source of an additive noise. A detailed analysis of this problem was given by Dobosz et al.\(^8\)^9. Finally, the signal amplitude at this pixel is recovered by means of

\[ B = \sqrt{b_1^2 + b_2^2}. \]  

(6)

The optical section is assembled from values of \( B \) at each pixel \((i,j)\).

3. Sine approximation algorithm characteristics

To characterize the sine approximation algorithm for image-reconstruction purposes, we considered four parameters which could affect the imaging results: (a) a systematic error of \( \delta \phi \) in the grating step size, \( \Delta \phi \), (b) random additive noise, (c) contributions of out-of-focus scattered light and (d) the dynamic range required for image reconstruction. The analysis was performed numerically by affecting the component \( \mathbf{S} \) in Eq. (5).

3.1. Phase step error influence on reconstructed image

A systematic error in the grating step size is common in structured-illumination, and is usually a result of a grating rotation (cosine error) or an improper oscillation frequency. The \( \delta \phi \) error arises from an incorrect \( \Delta \phi \) phase step value between acquired images. We limited the analysis below to such systematic errors only. The phase step error-analysis was performed using double number precision to show the general behavior for an incorrect step value. In this analysis, all considerations regarding dynamic range, signal level and discrete data character at the detector were neglected.

To analyze the influence of \( \delta \phi \) on the reconstructed amplitude, \( B \), the true signal sampling (\( \Delta \phi_S \)) was changed over a range 0 to 2 times (corresponding to an error of \( \pm \Delta \phi \)) \( \Delta \phi_S \), while the expected phase step, \( \Delta \phi \), remained constant. In this case, the \( \delta \phi \) error is equal to \( \Delta \phi_S - \Delta \phi \). This large (0 to 2) range was selected to demonstrate the oscillatory character of the influence of the phase step value on the reconstructed amplitude and to show the source of the residual grating. In practice, the user is likely to encounter much smaller

![Fig. 2. Imaging sequence at the CMOS/CCD detector for the structured-illumination technique. Five pixels are shown at the detector (D1-D5), and the illumination sequence is shown over three steps of the grating. The actual grating step size is \( \Delta \phi \), and the start phase of the image sequence at a particular pixel is \( \phi_0 \). For example, the initial phase for pixel D1 is 0. The image is reconstructed using the assumed step size of \( \Delta \phi \).](image-url)
systematic errors, for example in the range of ±5% of the expected step, \( \Delta \varphi \) (0.95 to 1.05 of \( \Delta \varphi \)). Additionally, a \( \varphi_0 \) change (in 0 to 2\( \pi \) range) was applied to monitor the reconstructed-amplitude results at different pixel locations. Figure 2 shows a portion of an imaging sequence at the CMOS/CCD detector with structured illumination for five selected sensor pixels. \( \Delta \varphi_0 \) is the actual shift of the structure over the detector between images, while \( \varphi_0 \) is the initial phase of the image sequence at a selected pixel, and is related to the relative position of the pixel and the grating. \( \Delta \varphi \) is the assumed step value used in reconstruction.

We found that the reconstructed amplitude, \( B \), depends strongly on the error between the actual step size and the step size assumed in the reconstruction. When the actual and assumed step sizes are not matched, the algorithm yields different amplitudes for different values of the initial phase. Figure 3 shows the oscillatory character of the reconstructed amplitude as a function of both the error in the sampling step, \( \Delta \varphi_0 \), and the initial phase, \( \varphi_0 \), at the pixel. Figure 3(a) shows a topographical projection of this relation. The reconstructed pixel amplitude with the incorrect step size, \( B \), has been normalized by the pixel amplitude reconstructed with the correct step size.

Figure 3(b) shows cross-sections of the normalized reconstructed amplitude for the locations marked with blue lines in Fig. 3(a) at sampling steps equal to 1.25 times and 1.50 times the true value, respectively. These cross-sections show a sinusoidal modulation of the reconstructed amplitude \( B \) across the image sensor when an incorrect sampling step size is used to compute the reconstructed image. For \( \Delta \varphi_0 \) close or equal to \( \Delta \varphi \) (\( \delta \varphi = 0 \)), the reconstructed amplitude \( B \) reaches a maximum value while its modulation is negligible. For 1.25\( \Delta \varphi \) (\( \delta \varphi = 0.25 \Delta \varphi \)) and 1.50\( \Delta \varphi \) (\( \delta \varphi = 0.50 \Delta \varphi \)), the ratio between \( B \) and its modulation decreases significantly. The consequence of signal modulation across the image sensor is a residual grating imprinted in the reconstructed image. This residual grating effect in the image is schematically shown at the right side of the Fig. 3(b). The object in this case consisted of two white circles. It should be noted, however, that there is no requirement to use any particular phase step value. It is enough to accurately determine the phase step and then use it for the reconstruction. This can be easily done by system calibration.

To quantify the residual grating effect in the reconstructed image we denote the amplitude modulation across \( B \) with the symbol \( B_M \) and the average value of \( B \) across the image (for different phase \( \varphi_0 \) in 0 to 2\( \pi \) range) by \( B_A \). Both \( B_M \) and \( B_A \) are determined for a fixed value of \( \Delta \varphi_0 \). The physical meaning of the quantity \( B_M \) is the signal modulation (amplitude of residual grating) across the CCD detector for a constant phase step, and that of \( B_A \) is the

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Fig. 3. (a) Topographical projection of the normalized reconstructed amplitude \( B \) as a function of the ratio of the real sampling step \( \Delta \varphi_0 \) to the assumed sampling step \( \Delta \varphi \) and phase \( \varphi_0 \) at the pixel (b) Cross-sections for locations marked with blue lines in (a) and sampling steps \( \Delta \varphi_0 \) equal to 1.25\( \Delta \varphi \) and 1.50\( \Delta \varphi \).
average signal across the CCD detector for a constant phase step. Therefore the ratio \( B/A/BM \) represents the “strength” of the residual grating effect in the image. Figure 4(a) presents the relation between the reconstructed amplitude and the strength of the residual grating effect in the reconstructed image. The values of the amplitude of \( B \), the modulation \( B_M \) and the ratio \( B/A/BM \) are plotted in blue, green and red, respectively.

![Figure 4(a)](image)

**Fig. 4.** (a) Reconstructed, normalized amplitude \( B \) (blue), amplitude of residual grating \( B_M \) (green), residual grating strength defined as the ratio of \( B/A/BM \) (red), (b) \( B/A/BM \) ratio for values of the step size within ±5% of the expected step size.

Figure 4(b) shows \( B/A/BM \) for a relatively small region centered around the correct sampling step size. Examining the ratio \( B/A/BM \) [ Fig. 4(b) ], one can see that if the sampling step \( \Delta \phi_S \) is within +5% of the expected \( \Delta \phi \) value, the amplitude of the residual grating pattern is less than 0.05 of the reconstructed amplitude \( B \). Therefore to minimize the residual grating from reconstructed images, the sampling step \( \Delta \phi \) must be determined with high accuracy.

As mentioned above, it is more important to know the exact phase step \( \Delta \phi_S \) than to achieve a specific \( \Delta \phi \) value. In practice, the structured-illumination imaging system can be calibrated before an imaging session or the sampling step can be found after acquiring raw-image data and using the Fourier transform. The Fourier transform approach has been applied previously for real-time shape measurements with the coherent fringe projection technique and sine approximation algorithm. Our previous experience shows that the sampling step may be determined with an accuracy of 1% or better.

It should be noted these simulations were performed using data acquired within exactly one signal oscillation across a pixel \( (N-1)\Delta \phi = 2\pi \). When acquiring data across more oscillations, one can expect more dense fluctuations in the reconstructed amplitude as the deviation between the actual and assumed step size increases. Even though the overall amplitude as a function of \( \Delta \phi_S \) may become more complicated, its character and, in particular, the behavior around the correct step size remain similar to the results presented above in Fig. 4(b).

### 3.2. Random noise

To investigate the influence of random noise (for example camera read noise or shot noise) on the reconstruction, random values were added to the signal \( S \) [ see Eq. (5) ]. Both uniform and normal random number distributions were applied. The observed effects of random noise were similar for both distributions, and therefore results shown here are limited to those for the uniform distribution only. Each numerical experiment was repeated 100 times for a different set of random perturbations to \( S \). Results presented below contain averaged curves resulting from these 100 computations.
Figure 5 presents the dependence of the reconstruction on SNR and number of images used in the reconstruction. SNR was defined as the ratio between the input signal amplitude and the amplitude of random noise added to this signal. The abscissa of Fig. 5 shows both the SNR and the normalized input amplitude. In these simulations, the input amplitude and SNR were varied in tandem, so that for input amplitude $B$ equal to 1.0, the SNR was also set to 1.0. Therefore, with a decrease in amplitude, the SNR also decreased. Figure 5(a) shows that, for low SNR, the reconstructed amplitude deviates from the true value of $B$ (red line). Additionally, this deviation is larger when a smaller number of images are used for the reconstruction. The number of signal oscillations – the number of $2\pi$ signal phase changes in the acquired data used for computations did not affect the reconstruction results.

To better illustrate the non-linear behavior of the reconstructed amplitude with increasing levels of random noise and for various numbers of images used in the reconstruction all curves presented in Fig. 5(a) were scaled to the range 0-1. The scaled data are shown in Fig. 5(b). However, for image processing purposes, this non-linearity is not critical since the most important is capability of resolving information across dynamic range (see section 3.3). Additionally, in practice the modulated signal is not stretched over the entire dynamic range of the camera but rather across a limited region. A significant part of the camera dynamic range is used by out-of-focus scattered light contributions. Therefore the local amplitude dependence may be close to linear and can be properly scaled.

For known imaging conditions (noise level, number of acquired images, dynamic range of modulated signal), this non-linearity may be compensated by incorporating scaling coefficients into the reconstruction procedure. For example, both the noise level for a defined level of out-of-focus scattered light and the dynamic range of the modulated signal (with structured illumination) can be determined. These values define the appropriate operating region in Fig. 5 and allow for correction of non-linearity.

3.3. Dynamic range vs. amplitude resolving capability

To assess the capability of resolving image amplitudes under the conditions of low SNR and low dynamic range, a synthetic test object was studied. The purpose of this test was to estimate what true dynamic range can be obtained using the sine approximation. Typically, when using structured illumination to image dense biological tissue samples in reflectance mode, the strength of the modulated signal is very limited, and it is necessary to avoid further data loss in the reconstruction.

The test object consisted of 24 adjacent pixels whose signals were distributed as shown in Fig. 6(a). The test-object signal amplitudes are denoted with green bars. The signal amplitude at each pixel in the test object was distributed over 4 gray levels only (i.e., pixel
values ranged from 0 to 3). The SNR for gray level 3 was 0.02, which is similar to experimental SNR we have obtained for structured illumination images of biological samples, where the shot noise plus out-of-focus contributions are usually 10 to 50 times stronger than the modulated signal. Only four images were used for reconstruction.

Under these test conditions, the reconstructed amplitude (red bars) match very well the input data amplitude (green bars): Instead of getting the exact input values of 1 and 2 we obtained 0.985 and 1.988 respectively [see the magnified portion of Fig. 6(a) marked with blue dotted frame and shown in Fig. 6(b)]. This deviation is most likely caused by the non-linear behavior for different SNR described in section 3.2. The observed differences are very small and effectively negligible. This small nonlinearity can be explained by small SNR variations. For a larger dynamic range and larger SNR changes, the user can expect stronger effects (suggestions how to eliminate non-linearity were given in section 3.2). The non-integer reconstruction results arise from the fact that after acquisition, data were converted to double floating point precision and all subsequent computations were performed with this representation.

The very important feature of this example is that we are able to reconstruct data for a modulated dynamic range as low as just two gray levels [for example pixels 9 and 10 in Fig. 6(b)] which was not possible for standard structured-illumination procedures introduced by Neil et al.\(^1\) It suggests that the sine approximation algorithm is capable of maintaining the entire dynamic range of the input data.

### 3.4. Out-of-focus scattered light contributions

Out-of-focus contributions primarily result in a spatially invariant image component at the image sensor. This spatially invariant component does not influence reconstruction results and can be neglected in computations. Therefore, to a first approximation, the influence of out-of-focus light may be neglected as long as there is enough dynamic range to reconstruct specimen optical sections. However, large magnitude of out-of-focus contributions (90-95% of well capacity of the sensor) may increase camera shot noise and introduce some effects similar to those discussed in section 3.2. For a detailed analysis, the raw-image noise level for different spatially invariant component values must be taken into consideration. During experimental studies we experienced out-of-focus contributions at the level of 70 to 90% of well capacity. The sine approximation algorithm was successful in removing this signal component.

### 3.5. Sine approximation algorithm summary

In summary the sine approximation algorithm exhibits the following characteristics:
- It does not require a specific sampling step size \( \Delta \phi \), however, this step must be stable over the time of the raw-image acquisition. The user may tune the optical-section reconstruction algorithm at will by adjusting the sampling step. In comparison, the classical three image algorithm\(^1\) requires fixed \( \frac{2}{3\pi} \) steps.

- A systematic phase step error \( \delta \phi \) between raw images causes fluctuations of the reconstruction result at different pixel locations. This leads to a residual grating in the reconstructed optical-section image. However, for small step errors the amplitude of residual grating also remains small ( ±10\% range). Additionally, for small error values, the dependence between the amplitude of the residual grating and the phase step error is linear. The conventional approach\(^1\) also experiences the residual grating problem due to incorrect phase step. However for the sine approximation the user is able to correct reconstruction results after acquisition which is not possible in the other case. To correct the residual grating effect, an actual phase step has to be found and used again in the reconstruction.

- For high SNR changes across the image (various pixels may have different modulated signal amplitude) a non-linear relation in reconstructed amplitudes can be observed. This effect may be potentially compensated by system calibration and the application of scaling factors. Moreover, the number of acquired images may be increased (especially for static samples) to decrease the non-linear effects. Again, the sine approximation compares favorably with the classical approach\(^1\).

- The optically sectioned image can be reconstructed with the same number of gray levels as were contributed to each raw image by the signal from the optical section (it is not possible in the conventional approach\(^1\)). This statement is true even if the optical-section signal is so low that it only spans two gray levels in each raw image.

4. Reflectance imaging with structured-illumination

4.1. Implementation of sine approximation algorithm – discussion

The structured-illumination technique offers a simple and inexpensive alternative to confocal microscopy if sufficient optical quality can be retained in the reconstructed image. Our long term goal is to make the structured illumination technique useful for real time in vivo imaging of biological tissues, which has the potential to improve real time diagnosis of a number of pathologic conditions. In vivo imaging requires a fast scanning and acquisition system. In this section, we discuss how we adjusted our experimental parameters so we could investigate the capabilities of this method for real time imaging of biological samples.

Ultimately, for real time imaging, the grating mechanism can continuously scan with a frequency of 25-100 Hz. This range of scanning frequencies affords a balance between sufficient image acquisition speed and reasonable integration times for the CMOS/CCD detector. We assumed an acquisition rate below 500 Hz and an integration time in the 2-8 ms range (currently our group is pursuing the design of 500 Hz CMOS detector for miniaturized microscope probe\(^13\)). To obtain an imaging rate of 8-16 frames/sec one will need to acquire approximately 16–64 images for one reconstructed section. Considering this scanning frequency (25-100 Hz), the CMOS acquisition rate (up to 500 Hz), and the number of raw images for required one optical section image (16-64), the number of samples/signal oscillation can range from 1.25 to 20 and the total number of acquired cycles ranged from −1 to 30.

To test the sine approximation algorithm on biological samples, the structured-illumination system based on a modified Zeiss inverted microscope (Axiovert 100M) equipped with a scanning amplitude grating was configured. Imaging experiments were carried out using a 63x, 0.95 NA water-immersion microscope objective, at a wavelength of 650 nm. The structure used in the system had a 100 \( \mu \)m period. It resulted in an axial resolution of ~0.5 \( \mu \)m. The imaging configuration was diffraction limited and the transverse system resolution was ~0.4 \( \mu \)m. The maximum field of view of the imaging system depended on the magnification and parameters of CCD camera (Hamamatsu ORCA C4742-95) and was
approximately 143 \( \mu \text{m} \times 108 \mu \text{m} \). However, for the purpose of this study a region of 512 x 512 pixels was selected which corresponded to field of view of 54 \( \mu \text{m} \times 54 \mu \text{m} \). It should be noted that to increase the system field of view, the combination of magnification and camera parameters (number of pixels and pixel size) should be optimized. Integration times and number of samples were adjusted according to the requirements discussed above (depending on the sample, integration times were in the range of 10-30 ms, the maximum total number of images/layer was 64, while the number of images/oscillation at a pixel ranged from 3 to 8). In this first reflectance experiment to acquire data, a stepper motor was applied to change the grating position. Therefore for one sample layer, a total acquisition time of 5 to 20 seconds was required. However, in the future, after switching to the resonant mode it will be possible to decrease this time to a fraction of a second.

4.2. Tissue phantom preparation

To demonstrate the structured-illumination technique in dense biological tissue, we prepared tissue phantoms containing multiple layers of epithelial cells which have optical, morphologic and molecular properties representative of normal and precancerous squamous epithelial tissue. In order to enhance the reflectance signal from cells, phantoms were labeled with molecular-specific contrast agents for reflectance imaging.

Here, we used contrast agents targeted against the epidermal growth factor receptor (EGFR), which is overexpressed in many cancers and precancers. The contrast agent consisted of gold nanoparticles to provide a bright source of backscattering, conjugated to anti-EGFR monoclonal antibodies to provide molecular-specificity. The gold nanoparticles were 20 nm in diameter with a peak scattering coefficient between 630 and 680 nm. The monoclonal antibodies are molecular specific to EGFR, which is a transmembrane Mr 170,000 glycoprotein that is overexpressed in epithelial precancers. Both colloidal gold and EGFR antibodies have been used clinically previously.

The tissue phantoms were prepared using SiHa cervical epithelial cancer cells and MDA-MB-435S breast epithelial cancer cells. The SiHa cells overexpress EGFR and provide a good model of neoplastic epithelium, while the MDA-MB-435S breast epithelial cells do not express EGFR, and in this regard, provide a good model of normal tissue. Phantoms of each epithelial cell line were prepared by resuspending cultured cells in a volume of buffered collagen type I to obtain a cell density of \( 10^8 \) cells/ml. Collagen-cell suspensions were plated into 6.5 mm diameter transwells and allowed to gel at 37°C. The prepared tissue phantoms were allowed to grow in DMEM plus 5% FBS for 24 hours so that they formed a highly dense structure consisting of multiple layers of epithelial cells. The phantoms were determined to have a thickness between 400 and 600 \( \mu \text{m} \). A solution of the anti-EGFR gold contrast agent was applied to the top surface of the phantom for 10 minutes; phantoms were then rinsed with phosphate buffered saline (PBS) and imaged. Labeled MDA-MB-435S phantoms which do not express EGFR were expected to give very little reflective signal compared to that observed with labeled SiHa phantoms. Labeled SiHa phantoms which overexpress EGFR were expected to show clear labeling of cell membranes. As a control experiment to test for non-specific binding, a separate set of phantoms were also labeled with 20 nm diameter gold nanoparticles conjugated to non-specific IgG antibody and imaged; very little reflectance signal was expected under these conditions.

4.3. Imaging results

Figure 7 presents imaging results for a phantom made of SiHa cervical cancer cells labeled with the anti-EGFR gold contrast agent. Figures 7(a), 7(b), and 7(c) show a conventional widefield reflectance microscope image, a structured-illumination raw image, and a reconstructed optical-section image, respectively. The reflectance from the colloidal gold particles can be appreciated at the cytoplasmic membrane of the SiHa cells which overexpress the EGF receptor. This characteristic “honeycomb” pattern is consistent with all of our previous results for this contrast agent using conventional confocal microscopy.
in Fig. 7(c) represents a layer 15–20 \( \mu m \) below the phantom surface. The image in Fig. 7(c) is one of 30 optical sections scanned with 1 \( \mu m \) axial increments through the tissue phantom. A “fly-through” all optical sections is presented in animation - 1.9MB. Figure 8 presents an image of the same layer as shown in Fig. 7(c) but reconstructed with classical structured illumination\(^1\) approach. The significant loss of dynamic range comparing with sine approximation algorithm is noticable. The reflectance signal intensity from these cells labeled with the anti-EGFR gold contrast agent antibody is much brighter than that with the non-specific control antibody (data not shown).

Figure 9 shows imaging results for the MDA-MD-435S phantom labeled with the anti-EGFR gold contrast agent. Figure 9(a) and 9(b) show a structured-illumination raw image and a reconstructed optical-section image, respectively. The image shown in Fig. 9(b) represents an optical section approximately 20 \( \mu m \) below the surface of the phantom. The image in Fig. 9(b) is one of 25 optical sections scanned with 2 \( \mu m \) axial increments through the tissue phantom. The resulting optically sectioned images of the 435 cells labeled with the anti-EGFR gold are very similar to the control images obtained with both the 435 cells and the SiHa cells labeled with the non-specific control antibody; the SiHa cells labeled with the specific antibody (Fig. 7) show much higher intensity as expected. The images in Fig. 9 (EGFR-negative cells labeled with the EGFR-specific contrast agent) provide an indication of the ability of structured illumination to image the native reflectance of tissue. In this case, we find that the image detail present in both structured illumination and confocal images (data not shown) compares favorably, as does the penetration depth. Since acetic acid was not applied.

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Fig. 7. Images of phantoms containing SiHa cervical cancer cells labeled with anti-EGFR gold conjugates. The field of view is 54 x 54 \( \mu m^2 \). The approximate depth of the imaged optical section is 15-20 \( \mu m \) below the phantom surface. Part (a) shows an inverted widefield reflectancemicroscope image. Part (b) shows a structured-illumination raw image. Part (c) shows a reconstructed optical-section image (animation - 1.9MB).

Fig. 8. Image of phantom containing SiHa cervical cancer cells labeled with anti-EGFR gold conjugates reconstructed with classical structured illumination algorithm.
to these cells, there is little detail present in either the confocal or structured illumination images.

4.4. Comparison with “gold standard” confocal microscopy

In order to compare our structured-illumination results with a “gold standard” imaging system, we collected confocal microscope images of the SiHa tissue phantom stained with the anti-EGFR gold colloid. The confocal microscope (Leica TCS-4D) was used in reflectance mode at 647 nm illumination with a 63×, NA = 0.95 water immersion objective. Figure 10 presents a confocal-microscope image taken at approximately 15 µm below the surface of the phantom. There is a very good match in terms of contrast and detail between the images presented in Fig. 7(c) (structured illumination) and Fig. 10 (confocal microscopy). It is worth noting that the structured-illumination microscope and the confocal microscope could image the same sample to a maximum depth of 50 µm. We found that this maximum depth was limited by the labeling technique rather than the performance of each instrument and its imaging modality. A “fly-through” all optical sections separated axially by 1 µm is attached in animation 2.3MB.

4.5. Quantitative image analysis

The reconstructed structured illumination images were analyzed quantitatively to determine whether image intensity parameters could be used to discriminate images of the phantoms...
representative of normal and neoplastic tissue. We compared the mean signal intensity in the vicinity of the cell membrane, since this is the area that is labeled with the anti-EGFR gold colloid. The mean reflectance intensity at the vicinity of the cell membrane was found for each of approximately five of the SiHa and 435S cell phantoms individually at a similar sample depth. Images from both phantoms were first multiplied by a single constant factor to enhance their brightness. The NIH freeware program ImageJ was then used to hand-segment the area of the cell membrane and calculate the mean reflectance value within the selected region. The average intensity of the cell reflectance signal was 62.5 gray-scale values for SiHa phantoms \( \bar{M}_{\text{neo}} = 62.5 \), substantially greater than the average 28.5 gray-scale reflectance signal for the MDA-MD-435S cell phantoms \( \bar{M}_{\text{norm}} = 28.5 \). The corresponding standard deviations were \( \sigma_{\text{neo}} = 4.9 \) and \( \sigma_{\text{norm}} = 3.5 \), respectively. The difference in the average membrane intensities is greater than the sum of the associated standard deviations, i.e.

\[
\left| \bar{M}_{\text{neo}} - \bar{M}_{\text{norm}} \right| > (\sigma_{\text{neo}} + \sigma_{\text{norm}}) : \left| 62.5 - 28.5 \right| = 34.0 > (4.9 + 3.5) = 8.4. \quad (7)
\]

5. Summary

In summary, we have demonstrated the feasibility of the sine approximation algorithm applied to structured-illumination imaging. Additionally to our best knowledge we are the first group to have successfully performed structured-illumination imaging in reflectance mode of optically dense tissue phantoms. Our results compare very favorably with “gold standard” confocal microscope images of the same phantom. We also showed that phantoms representative of the optical and molecular properties of neoplastic and normal tissue labeled with a simple optically active, molecular-specific contrast agent can be distinguished quantitatively using optical-section image data that we have collected and an image-analysis metric that is sensitive to the difference in labeling based on molecular biomarkers of precancer and cancer.

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