

Structured illumination-based super-resolution optical microscopy for hemato- and cyto-pathology applications

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Abstract. Structured illumination fluorescence microscopy utilizes interfering light and the moiré effect to enhance spatial resolution to about a half of that of conventional light microscopy, i.e. approximately 90 nm. In addition to the enhancement in the x and y directions, it also allows enhancement of resolution in the z - direction by the same factor of two (to approximately 220 nm), making it a powerful tool for 3-D morphology studies of fluorescently labeled cells or thin tissue sections. In this report, we applied this technique to several types of blood cells that are commonly seen in hematopathology. Compared with standard brightfield and ordinary fluorescence microscopy images, the 3-D morphology results clearly reveal the morphological features of different types of normal blood cells. We have also used this technique to evaluate morphologies of abnormal erythrocytes and compare them with those recorded on normal cells. The results give a very intuitive presentation of morphological structures of erythrocytes with great details. This research illustrates the potential of this technique to be used in hematology and cytopathology studies aimed at identifying nanometer-sized features that cannot be distinguished otherwise with conventional optical microscopy.

Keywords: Super-resolution microscopy, structured illumination, blood cells

1. Introduction

Light microscopy has been an important tool in pathology due to its non-invasive nature and other advantages, such as low cost, simpler processing, etc [1]. Traditionally, pathologists have relied on transmitted light microscopy, with samples often stained with hematoxylin, eosin (H&E), and other dyes with strong absorption of visible light and different affinities to specific cellular structures. Contrast of various structures

obtained in such a way arises from different colors rendered by absorption of the dye molecules staining different cellular structures. This method is simple and well developed for pathology applications, and atlases of various diseases are available for reference. However, since the contrast comes from absorption of light, it is hard to locate an absorption event along the direction of light propagation, i.e., the optical axis. Therefore, ordinary transmitted light microscopes have poor sectioning ability, resulting in 3-D structures represented as 2-D images. Thus, it is impossible to collect 3-D morphological images of cells or cellular structures (of the order of microns) with ordinary transmitted light microscopy. This is a big problem

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for pathologists who want to relate the morphology to functionality and reveal the interplay between the structure and function. To overcome this problem, pathologists often resort to cutting samples into thinner sections and assemble the 2-D images of these sections into montage sequences. The axial resolution obtain in this way is still limited to micrometer resolution (i.e., the thickness of the sections). With advances in microscopy and in immunohistochemistry, fluorescence microscopy provides an easier solution to this problem [1]. Unlike transmitted light microscopy, fluorescence microscopy techniques are based on photon emission, which can be traced back to a point source. Therefore, fluorescence microscopy can resolve points along the optical axis better than bright field transmitted light microscopy. Thus, it is feasible to get 3-D fluorescence images in a noninvasive way. Since eosin (and some other commonly used dyes in pathology) is fluorescent when excited with light at around 530 nm, it is convenient to utilize fluorescence microscopes to collect 3-D morphological images of eosin (or other fluorescent dyes) stained cells or cellular structures without complicated sample preparation.

Due to the wave nature of light, resolutions of optical microscopes are subject to a limit imposed by optical diffraction, known as Abbe's diffraction limit [2]. Because of this limit, ordinary fluorescence microscopes have lateral resolutions of ~ 250 nm and axial resolutions of ~ 1 μ m. Confocal fluorescence light microscopes further improve the ability of sectioning, making them capable of collecting morphological images with improved quality. However, in terms of lateral resolution, confocal light microscopy is still limited by the Abbe's diffraction limit, therefore its lateral resolution is not much better than that of ordinary microscopes. Meanwhile, in practice, the axial resolution of a confocal microscope is often more than twice of its lateral resolution [3].

While biology and medicine have significantly benefited from developments in optical microscopy, additional improvements are needed to address the need for more detailed information at nanometer scale, and the resolution of confocal microscopy often cannot meet these needs.

Recently, several techniques aimed at super resolution fluorescence microscopy have been developed [4, 5]. They have lateral resolutions of 20–120 nm, which are well beyond the optical resolution achieved with conventional microscopes. Historically, the first super resolution microscopy technique that has been devel-

oped is Stimulated Emission Depletion microscopy (STED), which, by manipulating the beam profile of a 'quenching' laser, reduces the effective emission volume to achieve a lateral resolution of tens of nm [6]. Shortly afterward, photoactivation based techniques (including Stochastic Optical Reconstruction microscopy (STORM) [7] and Photoactivated Localization Microscopy (PALM) [8]) were invented, which take advantages of localizing photoswitchable dyes (whose fluorescence status can be toggled with a UV laser beam) one by one to reach a lateral resolution as high as 20 nm. Meanwhile, Structured Illumination Microscopy (SIM) [9, 10] and some other techniques (such as Super-resolution Optical Fluctuation Imaging (SOFI) [11]) were also developed. While the resolution of SIM is not the highest among super-resolution techniques, this method is fast (acquisition times are comparable to that of a confocal microscope), contains a relatively simple laser system, has no special requirements for sample staining (the sample just needs to be fluorescent and stable), offers resolution improvement in all 3 dimensions and possibly high penetration depth of imaging (up to approximately 30 microns) [4, 5]. Since this report is not intended to elaborate on the pros and cons of various super-resolution microscopy techniques, readers who are interested at a detailed review and comparison of super resolution microscopy techniques are suggested to read references 4, 5 and references therein.

Structured illumination microscopes have lateral resolution of about a half of that of a conventional microscope and an axial resolution of less than half of that of a confocal microscope [10]. Thus, structured illumination microscopes are capable of revealing more details which otherwise could not be observed by conventional microscopes (including confocal microscopes). Therefore, this technique quickly attracted much attention since its invention, and has been successfully applied to study liver fenestration [12], membrane protein diffusion [13], cell apoptosis [14] and cortical construction during abscission [15]. In all these researches, SIM revealed rich information about structures or processes that could not be resolved by conventional microscopy.

As a science about structural alterations of cells and the consequences of the changes, pathology has a strong need for 3-D morphology images of cells and their ultrafine structures. Therefore, SIM has great potential applications in pathology, especially cytopathology. In this report, we have collected

fluorescent images of Wright-Giemsa stained blood cells using a structured illumination microscope (OMX Blaze, Applied Precision Inc., a GE Healthcare company.). The results clearly show the 3-D morphologies with great details of some common types of blood cells and their subcellular structures. We have also applied the technique to reveal the 3-D morphological differences between abnormal red blood cells and normal ones. This experiment illustrates the potential of super-resolution fluorescence microscopy in cytopathology.

2. Materials and methods

The most important property of a microscope is its resolution, i.e., its capability to resolve point-like objects that are close to each other. Most optical microscopes work in the far field region, i.e., at a distance larger than the wavelength of light, and consist of many optical components such as lenses, mirrors, prisms, etc. However, the resolution of an optical microscope is mostly determined by its objective lens. Besides of the objective lens, the configuration of illumination can also affect the resolution, as illustrated below.

2.1. Principle of structured illumination microscopy

In ordinary wide-field microscopes, the intensity of the excitation light is controlled to be uniform (or close to uniform) across the whole field of view (Fig. 1a). In such a configuration, fluorophores across the field of view are excited simultaneously and the fluorescence intensity reflects the number density of the fluorophores. The optical resolution obtained in this configuration, in which all optical components have limited sizes of clearance acting as various spatial low-pass filters, is limited by the light diffraction at the optical components, especially the microscope objective lens. In theory, the resolution (r) at the sample plane is a function of the fluorescence wavelength (λ) and the numerical aperture (NA) of the objective,

$$r = \frac{0.6\lambda}{NA}.$$

For light in the visible range and commonly used oil- or water-immersion objective lenses, this equation gives a lateral resolution of ~ 250 nm (for excitation light at 500 nm and NA of 1.2). This level of lateral resolution sometimes is sufficient; however, the resolution along

the z axis is often insufficient and the axial contrast is especially poor due to the out-of-focus background light. To enhance the axial resolution and contrast, confocal microscopes utilize a pinhole to reject out-of-focus background light, therefore effectively limiting the volume from which the collected signal is originated (Fig. 1a). This modification gives confocal microscopes the ability to section the samples along the optical axis, which helps slightly improve spatial resolution in all directions. However, the improvement comes with a sacrifice of signal intensity. To achieve the best spatial resolution, a very small pinhole has to be used, rejecting not only out-of-focus background light but also some of the wanted signals. In practice, a balance often has to be reached to meet the requirements of both the resolutions and signal intensity. As a result, effective axial resolution is often around 600 nm or more. Unlike confocal microscopy, SIM is a wide-field illumination technique; it adopts a different strategy to enhance both the axial and lateral resolutions. In a structured illumination microscope, a laser beam is split into three coherent beams that are simultaneously focused on the sample to generate a 3-D interference pattern (i.e., the illumination pattern). In the sample, the distribution of fluorophores form another pattern (i.e., the fluorophore pattern) with varying spatial frequencies, some of which are higher than (therefore blocked by) what is allowed by the optical transfer function (OTF) of the microscope optics. When the illumination pattern is superimposed on sample, its overlap with the fluorophores' pattern will generate beat frequencies that are called moiré patterns (Fig. 1b). Although the fringe spacing of the illumination pattern is still larger than the diffraction limit, the moiré pattern contains high spatial frequency information from sub-resolution features within the sample. Since the illumination fringe information, such as spacing, wavelength, and orientation, is known and controlled by the user, one can recover the spatial frequency of sub-resolution features of the sample from the moiré pattern. By rotating the orientation of the interference pattern (Fig. 1c, d and e) and modulating the phases of the pattern (i.e., moving the pattern), high spatial frequencies in all directions are collected. Using the obtained high frequency information, an image of the sample is then reconstructed with much higher spatial resolution. As an example, (Fig. 1f and g) show a comparison between an ordinary wide field image and a reconstructed structured illumination image of the same sample. From the comparison, it is clear that

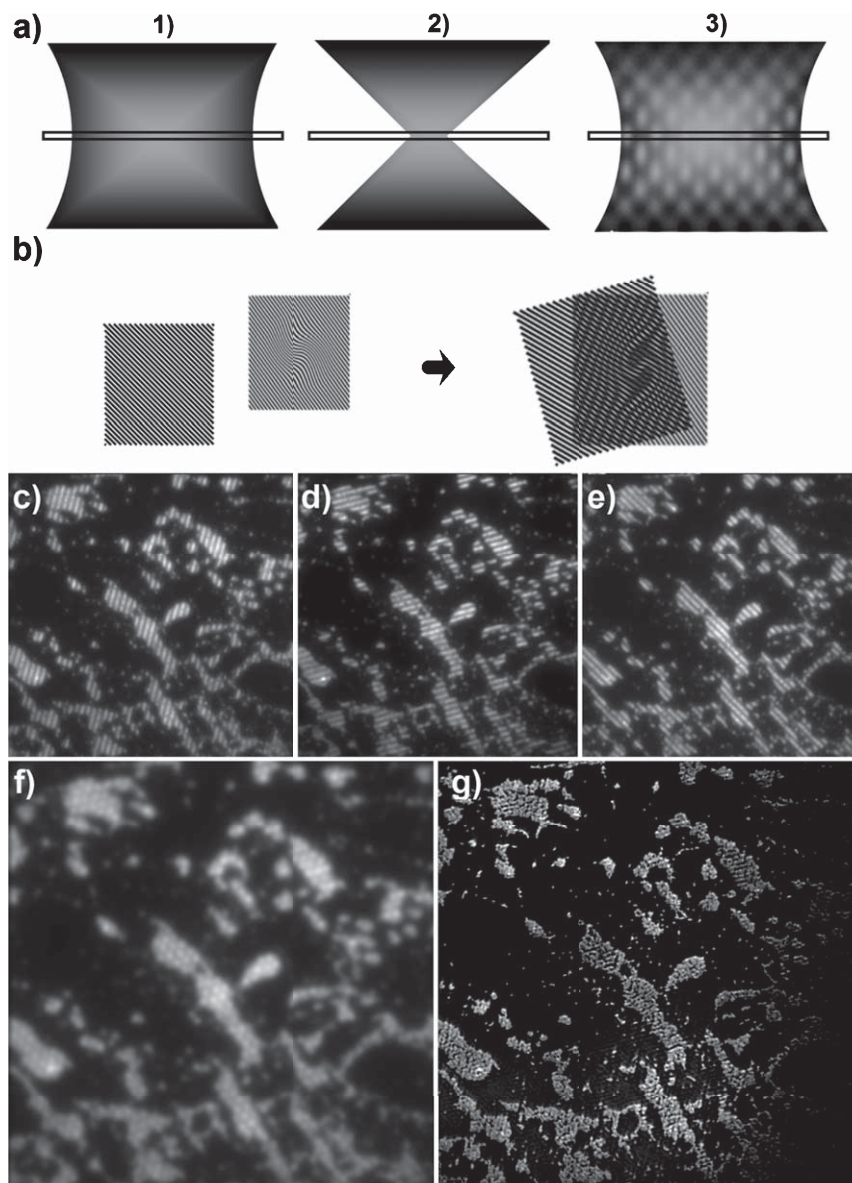


Fig. 1. (a) Different illumination profiles near the sample plane in (1) wide field; (2) confocal and (3) structured illumination configurations. (b) A moiré pattern generated from a high spatial frequency feature of the sample with a structured illumination pattern. (c, d, and e) Structured illumination patterns generated by interference laser beams at the sample plane at three different orientations. (f and g) An ordinary wide field illumination image and a reconstructed structured illumination image of the same sample.

SIM images have much higher spatial resolution than ordinary wide-field microscopy images.

2.2. Procedure

Normal blood smears were performed on glass slides. The blood smear slides were dyed with a

Wright-Giemsa stain consisting of Eosin Y, Methylene blue, and Azure B. The slides were then covered with #1.5 glass silica coverslips.

White light brightfield microscopy was performed using a DeltaVision Personal Deconvolution microscope (Applied Precision, Inc., a GE Healthcare company) with a 60X oil immersion objective (refrac-

tive index of the oil: 1.514). The color images were acquired through the eyepiece using a consumer camera equipped with a color CCD chip. Conventional fluorescence images were acquired using the same microscope and a Photometrics Coolsnap ES2 scientific camera attached to the side port. FITC (488 nm) and TRITC (555 nm) excitation channels were used. The fluorescence images were rendered using softWoRx 2.0 (Applied Precision, Inc.).

Structured illumination microscopy was performed with a superresolution microscope, an Optical Microscopy eXperimental (OMX) system v3.0 (Applied Precision, Inc., a GE Healthcare company). A 60X oil immersion objective with refractive index of 1.514 immersion oil was used in the experiment. To excite eosin, a 532 nm laser was used as the excitation source.

Super resolution fluorescence images were reconstructed using softWoRx 2.0 (Applied Precision, Inc.) with raw data collected with OMX. Reconstructed images were processed using Volocity 5.5.1 (Perkin Elmer Inc.) to generate 3-D images.

3. Results

Structured illumination microscopy has many potential applications in cyto-pathology in general and in hematology in particular. Hematology and cytopathology samples are often stained with eosin, which is fluorescent with a peak at ~ 550 nm when excited with light at 530 nm. Thus, it is convenient to apply SIM on eosin stained samples to survey the 3-D morphology of cells and subcellular structures. To illustrate this technique, we used SIM to investigate a few types of blood cells stained with Wright-Giemsa, which is the most common blood stain used in hematopathology, and contains eosin. The recorded images contain information about the spatial distribution of eosin within individual cells.

3.1. Imaging of normal blood cells and platelets

In Fig. 2, images of normal blood cells obtained with different microscopy techniques are juxtaposed to show the differences between techniques. The left column shows wide-field transmitted light microscopy images of a red blood cell, a monocyte, a neutrophil cell and a platelet (from top to bottom), collected with

a 60X objective. The middle column shows the corresponding fluorescence images of the cells, and the right column shows reconstructed 3-D morphological images of the cells. Under white light illumination, transmitted light images show different colors for nuclei and cytoplasm caused by absorption of the methylene blue and azure B dyes (staining the nucleus), and eosin (staining the cytoplasm). The color difference between nuclei and cytoplasm makes it easy to recognize different types of cells, explaining why this method is widely used in pathology. However, it is hard to discern fine details of cells with this method because often there is not enough contrast between levels of shades of staining (which is ultimately from absorption of staining dyes). This problem is even worse when the dyes are spatially mixed. Unlike brightfield microscopy where the background is very bright, making the absorption-based contrast difficult to increase, fluorescence microscopy utilizes fluorescence signal over a dark background to generate contrast. As a result, the contrast is very high. As shown in Fig. 2, it is obvious that images in the middle column (i.e., fluorescence images) have a better contrast than the left column (i.e., brightfield transmitted light images). The fluorescence intensity directly reflects the concentration of fluorophores that is associated with molecules, or structures under investigation. Since eosin is the only dye that can be excited with our laser in the experiments, the fluorescence reflected the distribution of eosin in the sample, which is mainly distributed within the cytoplasm. In these images, erythrocytes (Fig. 2, top row), which do not have nuclei, have cytoplasm filled with fluorescence while the platelets (Fig. 2, bottom row) and the nuclei of the WBCs (Fig. 2, middle rows), which are not eosinophilic, leave black areas in the images. Although the fluorescence images show some advantages over brightfield transmitted light images, due to the lack of a sectioning ability, the fluorescence images cannot reveal the real shapes of cells and cellular structures in three-dimensional space. As explained in the above section, structured illumination microscopy has superior resolution capability in both x -, y - and z - directions, enabling it to record the real shapes and structures in three-dimensional space. The right column shows reconstructed 3-D images of the same cells shown in the other two columns. The SIM images not only have better spatial resolution, but also a much better contrast. More importantly, the visualization of cellular structures in three dimensions makes it possible to discern many subtle differences

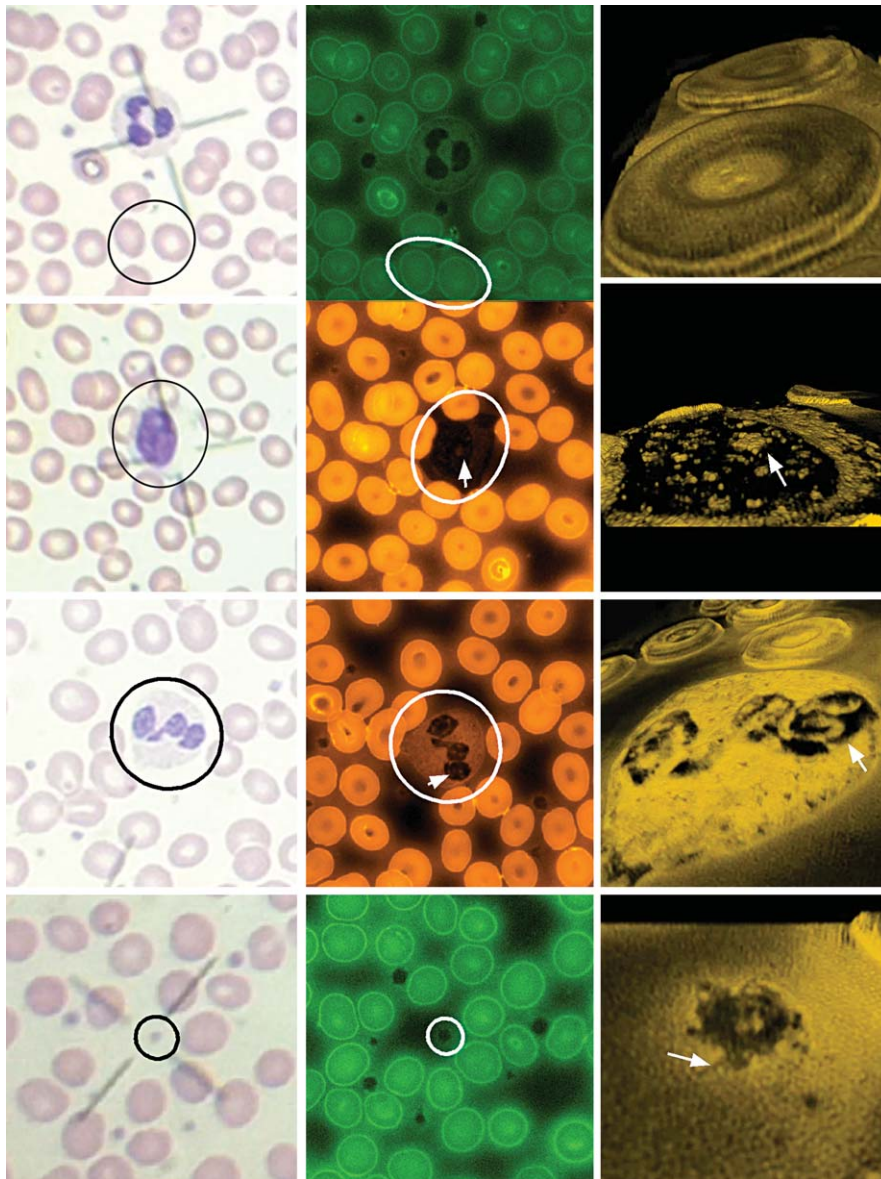


Fig. 2. Comparison of transmitted light (left column), fluorescence (middle column) and SIM (right column) images of red blood cells (top row), a monocyte cell (second row from the top), a neutrophil cell (third row from the top) and a platelet (bottom row). Note, the color in fluorescence images and in SIM images are pseudo-colors for rendering, not related to the color of the real fluorescence. The cells of interest are enclosed by circles, arrows point to features that are not well discerned in fluorescence images but in SIM images.

among cells. For example, the ultrafine structures of nuclei and cytoplasm of the WBCs (Fig. 2, middle rows) are clearly seen with details in the SIM images while they are very blurry in the transmitted light or conventional fluorescence images. The fine filaments

(pointed by the arrow) of platelets are also visible in SIM images while they are hardly discernible in other the two images. These comparisons clearly show the enhancement in resolution and the benefits of being able to visualize cellular structures in three dimensions.

3.2. Imaging of abnormal blood cells

To further explore the benefits of visualization of cellular structures in three dimensions, we investigated the morphologies of some abnormal erythrocytes (red blood cells), as shown in Fig. 3. The shape of RBCs is of interest to not only hematologists for its relation to its function and diseases [16], but also to biophysicists as a model to study membrane properties, such as elasticity [17–19]. Human RBCs in the blood smear are normally biconcave discocytes with a diameter of $\sim 8 \mu\text{m}$. Figure 3a shows an image of two normal blood cells, which have the standard biconcave morphology. However, under some circumstances, RBCs with abnormal shapes are found in patients with certain diseases. Therefore it is a common practice to check RBC morphologies in diagnostic laboratories. And it is scientifically meaningful to study the relation between abnormal RBC morphologies with diseases and genes. However, conventional brightfield transmitted light microscopy does not provide a 3-D morphology of RBCs. SIM images, on the contrary, revealed the 3-D morphologies of abnormal RBCs with ultrafine details. Figure 3b shows an image of an abnormal red blood cell, a stomatocyte. Instead of a circular pallor in a normal RBC, this cell has a slit in the middle of the cell. The slit is about 4 microns long and ~ 1 micron wide with corrugated side walls. Interestingly, the slit does not maintain a fixed width or shape from the top to the bottom. These fine details are otherwise not observable in ordinary brightfield microscopy. Although this disorder is thought to be the result of a membrane defect, the exact physiological mechanism is poorly understood and the molecular basis is unknown [20]. The morphological information collected with SIM is therefore helpful to improve the understanding of this abnormality. Figure 3c shows a codocyte, or a target cell, which looks like a shooting target under brightfield transmitted light microscopy. Under brightfield illumination, codocytes only appear to be target-like with a bull's-eye structure of shade. However, the SIM image shows that, in contrast to normal RBCs that have a uniform circular shallow pallor in the middle of the cells, this cell actually has a bulge in the middle of the shallow pallor. The cell in Fig. 3d is a spherocyte that has no central pallor at all and Fig. 3e shows a case of elliptocytes, cells that are elongated. The cell in Fig. 3d could be a spherocyte, which often is identified as erythrocytes without the characteristic pink ring-like periphery in ordinary brightfield microscopy.

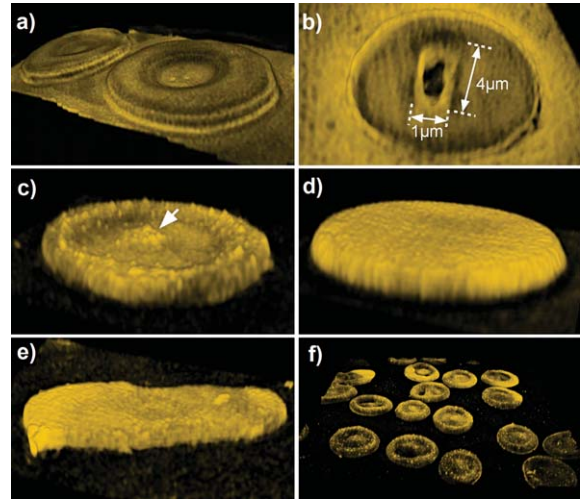


Fig. 3. 3-D morphological images of red blood cells. (a) Two normal red blood cells, (b) a stomatocyte, (c) a target cell, (d) a spherocyte, and (e) an elliptocyte. (f) A collection of abnormal red blood cells, in which knizocytes are also seen. Note the central bulging part in the target cell pointed by the arrow.

It is worth noting that although spherocytes are commonly referred to be spherical, the images shows that the cell on the smear slide is actually a flat plate-like cell with a thickness of 1 micron, instead of 2 microns for discocytes. The cell in Fig. 3e is an elliptocyte. It is interesting to note that in addition to the elongated shape, the cell surface is also very uneven compared to discocytes. It is not clear that the unevenness is related to the shape. Figure 3f shows a group of cells with various shapes in three dimensions.

4. Discussion

Structured illumination microscopy, as one of several super-resolution light microscopy techniques, has gained wide applications since its invention. Compared with super-resolution methods based on photoactivated localization (such as STORM or PALM), SIM is especially suitable for morphological studies. That is because photoactivation based super-resolution methods need to sequentially locate each individual fluorophores in order to record a super resolution image while, SIM, as a wide field method, captures information of all fluorophores at the same time. In morphological studies, the large quantities of densely populated fluorophores that are used to outline the

shape make the numeration of all the fluorophores impractical. In addition, photoactivation based super-resolution methods are best for 2-D imaging by their nature though they can be extended into 3-D. By comparison, SIM offers a 2x improvement in spatial resolution in all three dimensions, effectively reducing the volume by a factor of 8. More detailed comparison between SIM and other super resolution methods and conventional methods (including laser scanning confocal microscopy) can be found in literatures [21, 22]. Due to its novelty, SIM has not been widely used to study morphology of blood cells, but its capability has been demonstrated in a few studies about viral [23] or parasite infections of red blood cells [24] and immune responses of some white blood cells [25, 26], in which SIM revealed critical details of the process of infection or immune response that were otherwise not discerned.

In this paper we studied smeared blood samples with Wright-Giemsa staining. Although other fluorophores and methods of collecting the cells other than simple smearing are available, we adopted this sample preparation to demonstrate the compatibility of SIM to pathology, where morphology of cells at high spatial resolution is important for diagnosis. Eosin has been used in pathology for decades, mostly in combination with transmission microscopy. Its fluorescence properties and their use in pathology have also been well documented [27–30], but so far, despite the potential of their use for clinical applications, eosin fluorescence is not widely used by pathologists. By using eosin (and not other specialized photoactivable or photoswitchable dyes) in combination with SIM, pathologists can observe and evaluate the same sample that has been examined in transmission microscopy. Thus a more complete picture of the sample can be obtained.

5. Conclusions

Compared with conventional light microscopy, Structured illumination microscopy has doubled the resolving power in both planetary and axial directions. With its superior optical sectioning capability and its compatibility to work with eosin, a commonly used staining dye in pathology, SIM can conveniently collect 3-D morphology images of cells and subcellular structures with rich detailed information. Thus it has great potential in hematology and pathology.

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