Quantitative Label-Free Sperm Imaging by Means of Transport of Intensity

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ABSTRACT

Most living cells are optically transparent which makes it difficult to visualize them under bright field microscopy. Use of contrast agents or markers and staining procedures are often followed to observe these cells. However, most of these staining agents are toxic and not applicable for live cell imaging. In the last decade, quantitative phase imaging has become an indispensable tool for morphological characterization of the phase objects without any markers. In this paper, we report noninterferometric quantitative phase imaging of live sperm cells by solving transport of intensity equations with recorded intensity measurements along optical axis on a commercial bright field microscope.

Keywords: Quantitative phase microscopy, live sperm imaging, Transport of intensity equation, label free imaging.

1. INTRODUCTION

Quantitative imaging of sperm morphology and DNA integrity in sperms and has been a challenge to date for the biomedical imaging community [1-3]. To address this challenge, this paper uses a computational imaging technique based on transport of intensity equations (TIE) on diffracted images captured several micrometers apart from the image in focus of sperm cells in its native state without any staining procedures.

Among the various techniques for assisted reproduction technology (ART) currently available, In vitro fertilization (IVF) is the one that offers the best pregnancy rates for the treatment of infertility [4]. The semen quality parameters recognized as vital for assessment of fertility are sperm concentration, motility and morphology [5-7]. Semen analysis is widely used diagnostic tool to assess male reproductive health. The most important parameters measured in a semen analysis are the morphology, motility and the concentration of the sperm [8]. Thus, abnormal sperm features are the most common important indicators for male infertility [9]. Characterization of Sperm defects is very important in selection of sperms for assisted reproduction techniques. The shape of a sperm cell is an important determinant of its fertilizability because the egg is enclosed in a protein coat called the zona pellucida (ZP). The first task of ZP is to select the sperm that will fertilize the egg. The ZP's selection is based on the shape of the sperm head. In order to pass through the ZP, the sperm must be vigorously motile and the sperm head must be a symmetrical, and oval shape of the appropriate size. Sperms possessing heads that are irregular in shape, too round, too long, too big or too small are prevented from passing through the ZP. For this reason, there is a growing interest in understanding morphological alterations in spermatozoa.

The current techniques for characterization of spermatozoa are time consuming and destructive in nature. Hence, a non-destructive, and label-free method independent of environmental conditions (such as, temperature, pH level, and duration) avoiding any alteration of the vitality of the analyzed sperm would be highly helpful in the characterization of the semen. While spermatozoa are essentially transparent, and almost invisible when observed in optical bright-field microscopy, sperm cells have a different refractive index than the surrounding medium, the phase of the light transmitted through the sperm cell registers this modulation in refractive index. A qualitative visualization of this phase contrast may be obtained by phase contrast interference microscopy (phase contrast or Nomarski/Zernike interferential contrast microscopy) [10-11]. However these techniques cannot quantitatively estimate the phase of the cells. Hence characterization of sperm cells using these techniques will not be sufficient for a proper morphological assessment according to WHO criteria [12]. The possibility to use quantitative morphological analysis to provide a better understanding of the sperm behavior was reported by Coppola et al [13-14], Crha I et al [15] and Shaked et al [16]. However, most commonly used quantitative phase imaging techniques are based on interferometry and would require additional resources like costly coherent light sources, heavy

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computations and complicated experimental set-ups. In order to overcome these intrinsic limitations several approaches have been recently developed. In this context, we are proposing an optical high-resolution imaging approach that is a label-free, noncontact, non-invasive method that allows reconstruction of 3-D phase of live spermatozoa cells by recording two defocus images along the optical axis from the image in focus. In the last decade TIE has been successfully applied for real-time 3-D metrology in transmission electron microscopy (TEM) [17-18], micro lens characterization [19], recognition of bio organisms [20-21], and in X-ray imaging [22]. Non-interferometric quantitative phase imaging can be realized by transport-of-intensity equations (TIE), which was derived in 1983 by Teague [23-24] with the idea of relating measured intensities for phase quantification. It uses an elliptic partial differential equation called TIE. The main idea was that, when a plane wave is being illuminated by a phase objects like sperm cells, wavefronts from the light source are being modulated by the object and the object can be reconstructed by capturing these intensities at different defocused planes. Object can be reconstructed exactly if the defocused distance is optimal [25]. TIE imaging have been increasingly investigated during recent years due to its unique advantages over interferometric techniques [26-27], TIE is a non-interferometric imaging modality, and works with partially coherent light [28], computationally simple [29], it does not require phase unwrapping [30]. Despite these evident merits and great improvements of TIE, this technique has still not gained much attention and widespread applications as interferometric techniques in the field of quantitative phase microscopy. The use of TIE in the field of quantitative phase imaging of biological samples with nanometric precision and high SNR is highly promising. In this work we have extended the application of TIE to image live sperm cells with nanometric sensitivity in 3D using a commercially available bright-field microscope.

In this paper, we report a non interferometric quantitative phase imaging technique for live cell imaging of sperms from a regular bright-field microscope using the principle of transport of intensity equations (TIE). We report the results of some preliminary studies that illustrate the unique potential of transport of intensity equations (TIE) imaging to provide morphological information related to DNA content of sperm cells. The possibility to add the third dimension to morphological imaging of sperms can provide a better understanding of the sperm behavior with respect to male infertility[31-32].In this work we record three bright field images; one in focus, and two out of focus (+z, -z) images and implement the TIE algorithm to retrieve the 3-D phase information. Experimental results demonstrate the capability of this technique in 3-D volume estimation of sperm cells and characterization of sperm morphology with nanometric depth sensitivity. We also quantify the sperm parameters like acrosome area, head length, width and tail length. This real-time imaging technique will be highly promising for imaging phase samples without any preprocessing. This approach enables the characterization of live specimen, and on the other hand, allows both reducing the size of the mass storage devices required for image saving and achieving a fast image transfer. This technique shows high potential as a promising candidate for real time characterization of sperm cells without any sample preparation steps and identifying the anomalies associated with the sperm cells as per WHO standards [12]. In the following sections (2 and 3), principle and operation of TIE will be described followed by the materials and methods used for spermatozoa preparation. We discuss the results obtained by the TIE approach is in Section 4, which is followed by conclusions.

2. TRANSPORT OF INTENSITY EQUATION

2.1 Methodology and Experimental Set-up for TIE

The TIE is a Poisson type equation which relates a modified Laplacian of the phase of the wave to the intensity variation along the optical axis in absence of singularities in the in focus plane. The main problem to solve TIE equation is unknown boundary conditions. Which was bypassed by Fast Fourier Transform (FFT) based method by solving the TIE non-iteratively in the frequency domain with implicit assumed periodic boundary conditions.

A scalar field u(x, y) at a certain plane (image plane) can be represented in terms of amplitude and phase as

$$u(x,y) = \sqrt{I(x,y)}\exp(i\varphi(x,y)) \tag{1}$$

Where I is the Intensity and $\varphi(x,y)$ is the spatial distribution of phase. As we have mentioned above TIE uses only object field intensities at multiple axially displaced planes without any interference, we have recorded through focus intensities of sperm cells in the microscope. Then solved TIE for determining the object-plane phase from the first derivative of intensity in the near Fresnel region [25-26].

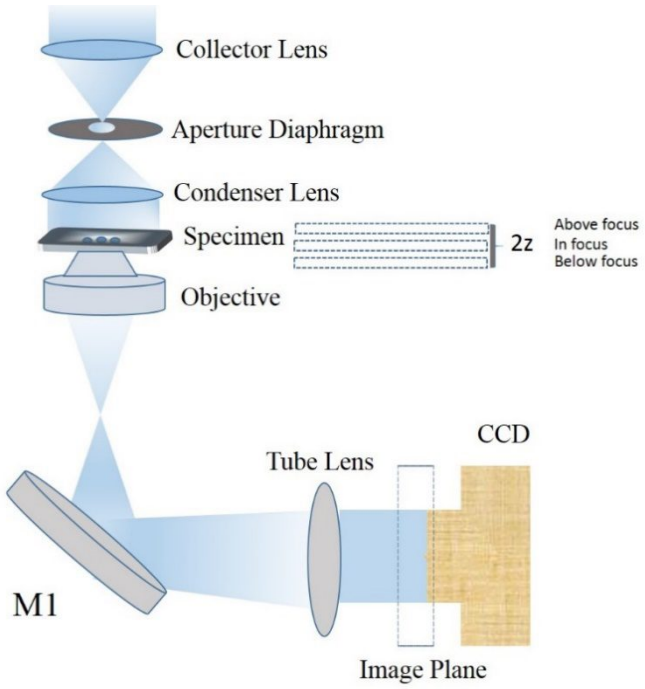


Figure: 1 configuration Bright field microscopy

Under the paraxial approximation the propagation follows Transport of intensity equation as follows [19].

$$\frac{\partial I(x,y)}{\partial z} = -\frac{\lambda}{2\pi}\nabla.\left(I\nabla\varphi(x,y)\right) \tag{2}$$

Where λ is the spectrally weighted mean wavelength. The detailed theory of TIE formalism has been explained in cited references and is excluded from this paper. The above equation relates the through-focus (z axis) intensity measurements to the two dimensional phase distribution. So with knowledge of the intensity distribution and its axial derivative (i.e., derivative along z) one can find information about the phase distribution. The intensity distribution can be directly measured at the image plane of the microscope and its z-derivative is obtained by defocusing the image slightly in both the positive and negative z-directions. A formal solution to retrieve the phase $\varphi(x,y)$ from eq. (2) is given by [33]

$$\varphi(x,y) = \nabla^{-2}\left[\nabla.\left[\frac{1}{I(x,y)}\nabla\left[\nabla^{-2}\left(-\frac{2\pi}{\lambda}\frac{\partial I(x,y)}{\partial z}\right)\right]\right]\right] \tag{3}$$

Where I(x, y) is the in focus image Intensity and $\nabla_{\perp}^{-2}$ is the inverse Laplacian operator and the intensity I is assumed to be strictly positive (i.e. there should not be any zeros in the measured intensity information within the field of view). The inverse Laplacian is solved by Fourier Transform method [33]. The solution is sensitive to boundary constraints, and can be solved using Fourier transform that assumes periodic boundary conditions.

Transport of intensity equation is a phase retrieval algorithm, which allows for a fully quantitative measurement of the cell optical thickness (i.e., the product of the refractive index and the physical thickness) on all the sperm

spatial points. This method requires a lower illumination power and presents high throughput because capturing is done in through axial measurements and without scanning.

The thickness of the object h(x, y) is calculated from the calculated phase as

$$h(x, y) = \frac{\lambda}{2\pi\Delta n}\Delta\varphi \tag{4}$$

Where Δn is the refractive index difference between the object and surrounding medium and we have considered a constant refractive index of $1.35, \Delta\varphi$ is the phase difference between the object and surrounding medium.

3. MATERIALS AND METHODS

Patients visiting the infertility clinic for fertility evaluation participated in the study. After formal approvals from Institutional Ethical Committee (UH/IEC/2014/114), semen samples were collected as per WHO approved protocols [12]. After liquefaction, routine semen analysis was performed according to WHO criteria [12]. Followed by determination of seminal volume in a graduated tube, sperm concentration was assessed by conventional method using Makler counting chamber (Sefi Medical Instruments, Israel) and expressed in millions/ml. The sperm motility was assessed in atleast 100 sperm and expressed as percent of motile sperm. Sperm morphology was assessed by Shorr staining and sperm viability by Eosin-Nigrosin stain.

The schematic of the experiment is shown in fig.1. Imaging is carried out on a regular inverted bright-field microscope (Carl Zeiss Axio-observer^{TM}) with a microscope objective of 63X with NA=1.41. The sperm cells are initially imaged using a tungsten halogen light source having central wavelength of 552 nm for bright-field imaging. Three bright field images of the objects are acquired with the help of a CMOS camera (1024 X 1388 pixels, size: 6.45 μm) for the phase retrieval using eqn 3. We recorded three images, one image in focus and two defocused images at optimal defocus ± 2 micrometers. The phase is determined by solving the TIE from eqn. 3 [31] on a Matlab^{TM} platform with the help of Fourier transform. The reconstructed phase has errors as mentioned in Ref. [31] and appropriate corrections are made on the reconstructed phase using a cubic spline interpolation method that calculates the unevenness in the background of the phase image with user-defined sampling points. Using these sampling points, the algorithm estimates the noise in the reconstructed phase image. The accuracy of this noise estimation depends on the number of sampling points. This estimated noise is subtracted from the reconstructed object plane phase, to obtain the actual phase of the object wave.

4. RESULTS AND CONCLUSIONS

From the reconstructed phase images of sperms, we can see that sperm cells are having head thickness of around 5μm, head length of 3-5μm. The DNA integrity with the sperm head is analyzed based on assumption of nucleus is having higher refractive index than the other regions as evident in the figs.3. (a)- (l). The results from the preliminary studies are summarized in table 1. As presented, we can analyze the 3-D volume, size and shape of the sperm head, the midpiece and tail segment. The results show a depth sensitivity of 100 nm at the tail segment. Any anomalies like vacuoles, inflammation in sperm head can be clearly distinguished in the 3-D phase images in fig. 2 and fig.3.

We have demonstrated a relatively simple technique to characterize live spermatozoa cells with the help of a regular bright-field microscope without any time consuming sample preparations and complicated staining procedures. This real-time imaging technique would be highly promising for clinical sperm analysis. It is very important to analyze morphological alterations of spermatozoa in their nearly physiological environment as staining procedures reduces the vitality of the cells making them useless for insemination. While the preliminary results are highly promising, experiments are being carried out in comparing and correlating the observations with histology.

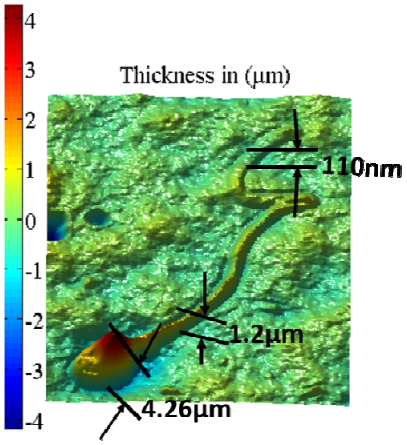
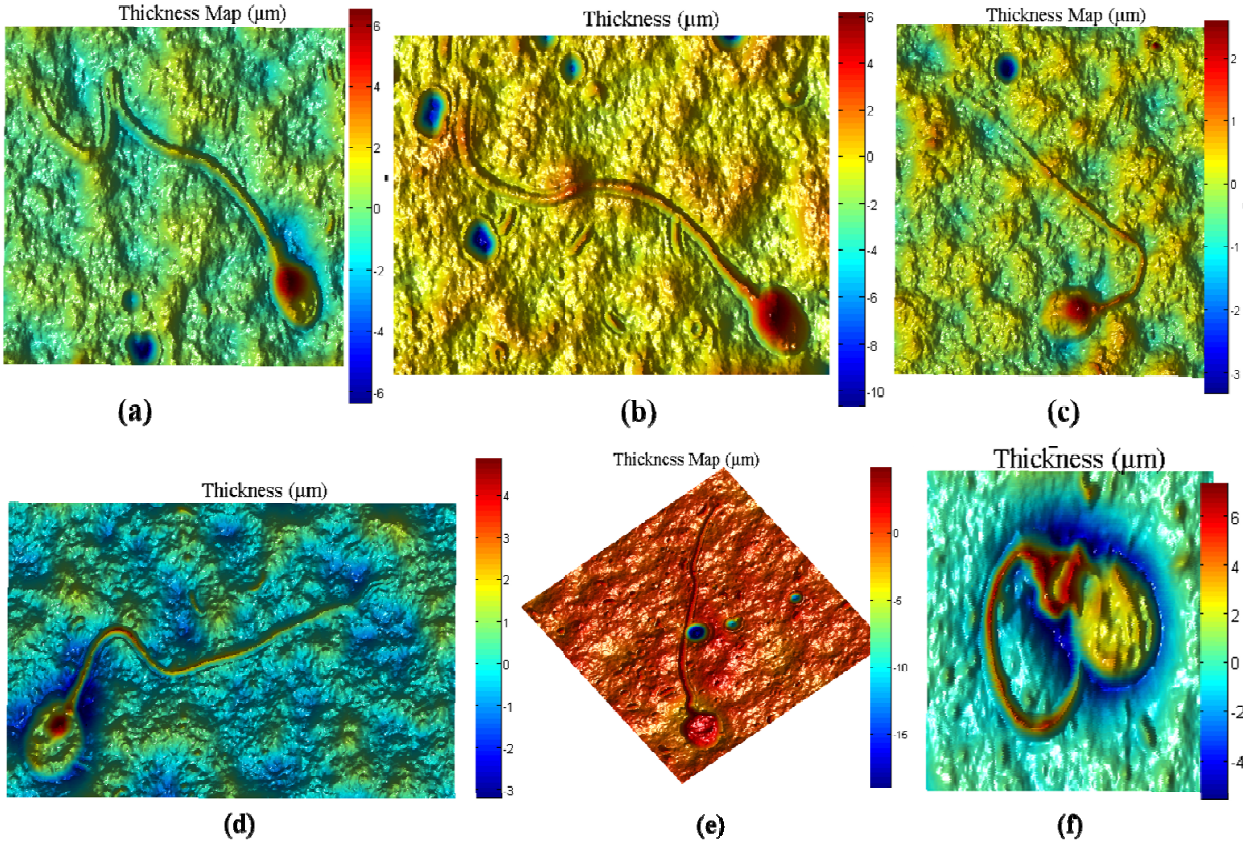


Figure.2. sperm 3D reconstruction



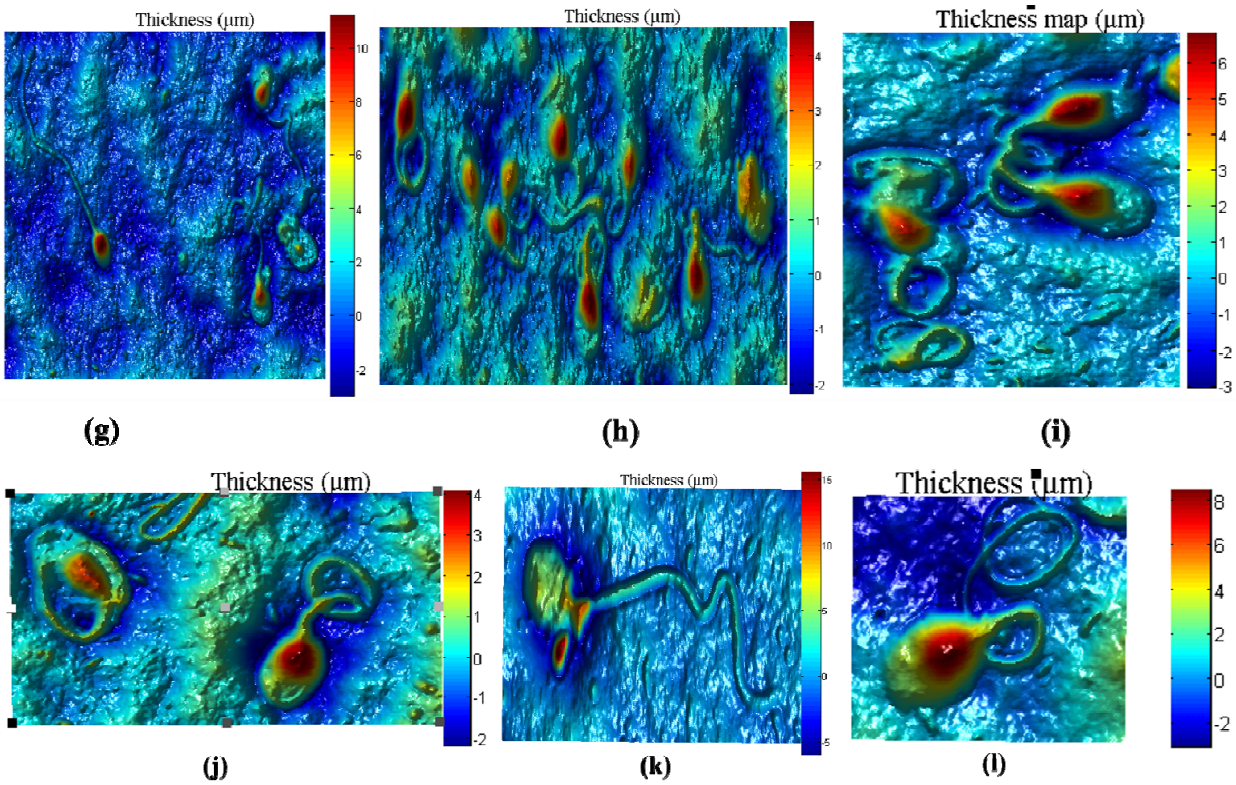


Figure.3. sperm defects reconstruction

Sperm Figure 3	**Head**	**Midpiece**	**Tail**
Fig. a	Normal	Normal	Bent
Fig. b	Normal	Normal	Normal
Fig. c	Normal	Normal	Little bent
Fig. d	We can see vacuoles (normal)	Normal	Normal
Fig. e	Normal	Normal	Normal
Fig. f	Abnormal	Abnormal	abnormal
Fig. g	One isolated sperm is good and the remaining are abnormal	Except the one other are abnormal	Except the one remaining are abnormal
Fig. h	Except 1 or 2 remaining heads are normal	Abnormal	abnormal
Fig. i	One is abnormal and the others are normal	Abnormal	abnormal
Fig. j	One is normal and the other is abnormal	Abnormal	bent
Fig. k	Abnormal	Normal	Bent
Fig. l	Normal	Not good	bent

Table.1 Description of images

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