

Review Article

Label-free Quantitative Imaging of Sperm for *In Vitro* Fertilization using Interferometric Phase Microscopy

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Abstract

Accurate morphological evaluation via imaging of sperm cells is a critical step in both routine sperm analysis and sperm cell selection for *in vitro* fertilization. In this paper, we review the significant advantages of interferometric phase microscopy (IPM), also called digital holographic microscopy, for label-free sperm imaging in fertility clinics. We first review the current state of the art of label-free sperm cell imaging for *in vitro* fertilization, including bright-field, Zernike phase contrast, differential interference contrast (DIC), Hoffman modulation contrast and polarized light microscopy, as well as intracytoplasmic morphologically selected sperm injection (IMSI). Using experimental demonstrations, we compare the limitations of these techniques for label-free sperm imaging. IPM and its advantages are then discussed in detail. These include the ability to receive quantitative data regarding the 3D structure of sperm cells, as well as data concerning sperm biomass and morphology without labeling. The current gap to clinical use is discussed, as well as the possible solution of employing compact and simple holographic modules, which may allow holographic imaging to reach the clinic in the near future.

Keywords: Holography; Imaging; Sperm Analysis

Introduction

Since 1978, when the first successful *in vitro* fertilization (IVF) was performed, great strides have been made in improving and refining the techniques and procedures involved. However, while great advances have been made in fertilization, cultivation and implantation methods [1], relatively little progress has been made in improving the imaging techniques used to assess human sperm and ova for IVF. Since exogenous staining cannot be used in IVF, due to the possibility of damaging the viability of the sperm, phase contrast imaging techniques are used. The phase property of light is related to how much light is delayed when passing through the sample. Cameras and the observer eye, however, are not fast enough to distinguish these small light delays. Therefore, phase contrast imaging methods are employed in order to convert these light delays into intensity changes. Modern IVF laboratories still employ techniques such as Zernike phase contrast microscopy, Hoffman modulation contrast microscopy or differential interference contrast microscopy [2], techniques that have been used for decades and provide only 2D information regarding the imaged cells, as well as distinctive imaging aberrations that might occlude important morphological details. Interferometric phase microscopy (IPM), on the other hand, is able to provide a quantitative light delay map for all spatial points of the imaged sperm. However, up till now IPM, also known as holography has been too complicated and expensive to be implemented in clinics. Recently, advances in holographic imaging have been made that may soon lead to the advent of new holographic imaging methods at the clinical level. These holographic images furnish the clinician with new quantitative data regarding the 3D structure of the cell, cell dry mass, as well as other new metrics. The purpose of this article is to present information concerning the current state of the art, as well as the new holographic methods currently in development.

Current Imaging Techniques for IVF

Bright-field

Bright-field microscopy is the most basic microscopy technique, in which the sample is illuminated with white (multicolor) light, with the contrast resulting from the varying optical absorbance of the sample. Regions that are more opaque will absorb more light and appear darker than more transparent regions. Exogenous contrast agents may also be

used to stain the sperm and function as a source of contrast. However, while bright-field microscopy is regularly used when analyzing stained sperm cells in label-based microscopy, its use is limited in IVF where labelling of the cells is not possible. Without labelling, sperm cells are mostly transparent and bright-field contrast is very poor [3]. Figure 1(A) shows a stain-free bright-field image of a sperm cell. As shown in this image, when not using staining, only the cell edges are visible, and there is no internal imaging contrast. This does not allow the performance of accurate morphological sperm assays for IVF.

Zernike phase contrast

Zernike phase contrast microscopy was the first imaging method to enable the utilization of phase differences as a source of contrast. It converts the light delay accrued when passing through the sample into intensity changes that can be recorded by a camera or visualized by the observer directly. This conversion from light delay to intensity is done by recording the interference of two light beams. In this case, the interference is created between the light that passes through the sample without changing its direction and the light that changes its direction due to interacting with the sample edges, also known as diffracted light. This is done by using a ring aperture to illuminate the sample with a hollow cone of illumination. The diffracted light will travel a different path than that of the non-diffracted light, allowing these two light beams to be modulated and interfered through use of a special objective containing a phase modulating ring aperture known as a phase plate [4]. Figure 2 shows a block diagram of this optical imaging system.

In the resulting phase contrast image, regions of the sample are made brighter or darker depending on whether positive or negative

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phase modification was performed. In the positive mode, regions or objects with higher refractive indices than the surrounding medium are darker in the image and edges of objects are differentially enhanced. This produces an image that is essentially a density map, with edges possessing enhanced contrast. The most significant drawback to this method is that it produces a halo around objects such as cells, caused by some of the diffracted light entering the ring in the phase plate [4]. Figure 1(B) shows the same sperm cell imaged in Figure 1(A), but now imaged with positive Zernike phase contrast microscopy. As can be seen in this image the acrosome appears to be brighter than the nucleus, and the border between these two regions is clearly visible. A typical halo aberration can be seen surrounding the entire cell.

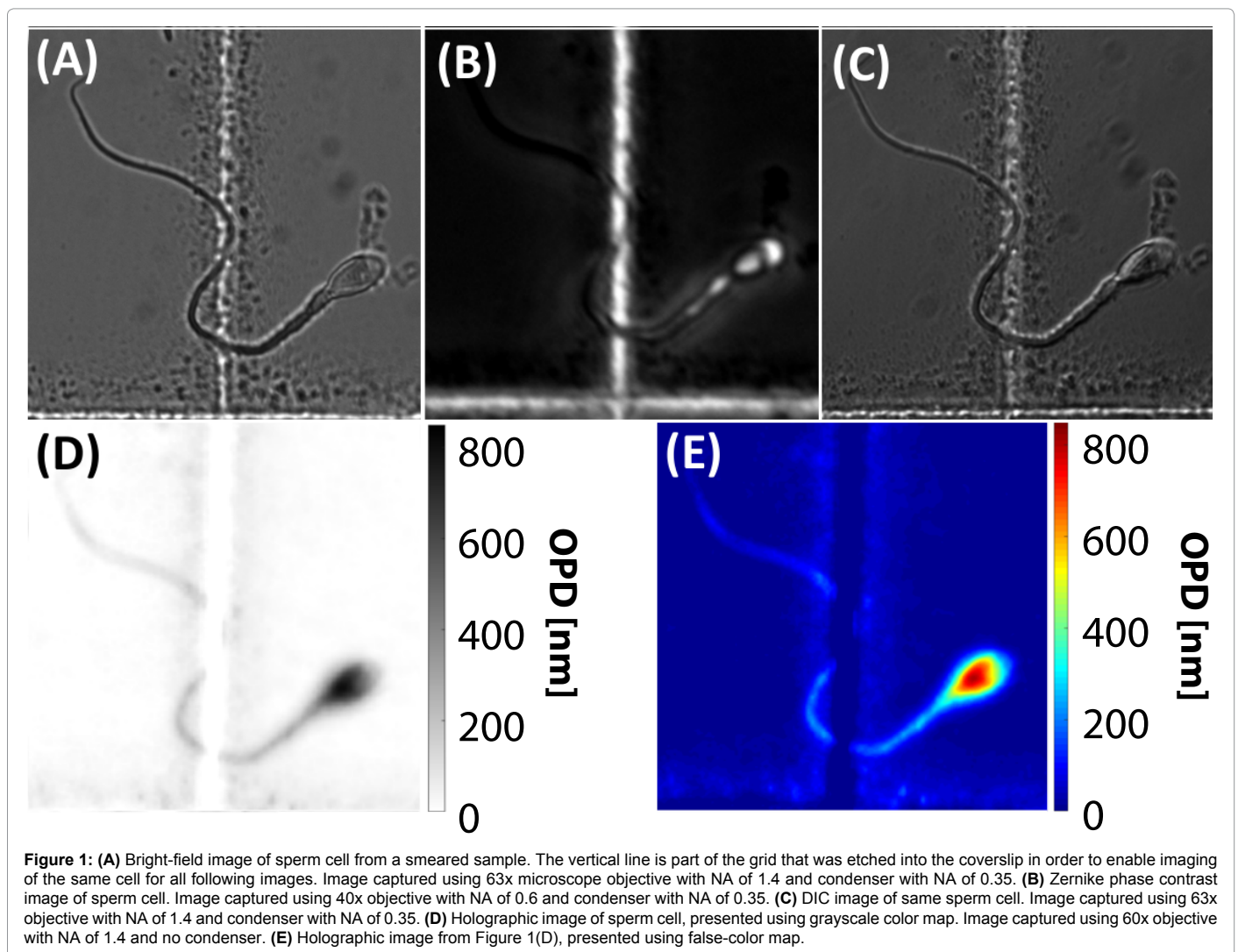
While this imaging method is old, having been invented in the 1930s, it still sees great use due to its simplicity. Some commercially available automated sperm analyzers are based on methods utilizing Zernike phase contrast, such as the TruMorph system by PROiSER R+D [5].

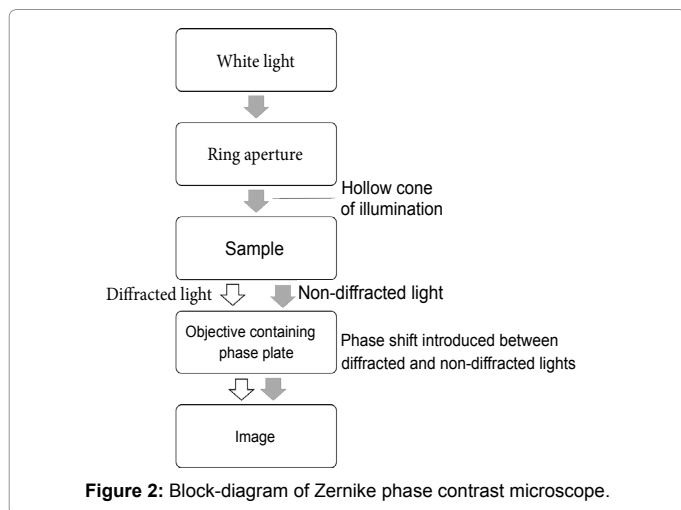
Differential interference contrast (DIC)

Among the most popular of imaging techniques for IVF is DIC. In this phase recording technique the interference is recorded between two light beams passing through the sample in close proximity, thus creating a pseudo-3D effect near cell and organelle edges. These beams

are laterally displaced and perpendicularly polarized by a Wollaston prism placed before the condenser, and the resulting slightly-shifted images of the sample are subsequently overlaid by a second Wollaston prism placed after the objective. On the basis of the magnitude of the lateral displacement between the two beams, known as the shear distance, we receive an interference image where intensity is determined by the phase difference between the displaced beams. A block diagram of this optical system is shown in Figure 3. The resulting interference image has a quasi-3D appearance, where regions of differing optical thicknesses possess edges that stand out as either brighter or darker than their surroundings. The width and intensity of these edges is highly dependent on the shear distance used [6].

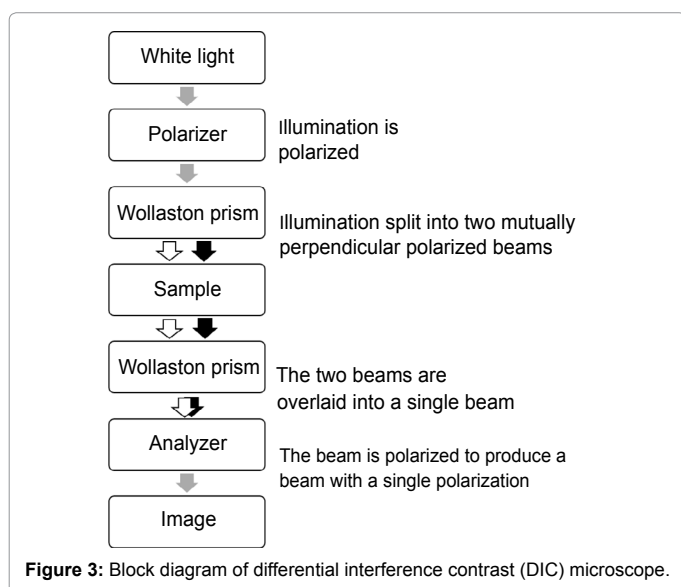
In this manner DIC improves contrast by enhancing the edges in the sample. Additionally, DIC improves contrast by using the full objective and condenser apertures, allowing the highest possible numerical aperture (NA) for a given microscope. Z-resolution is improved as well, as the main contrast of the DIC image is found within the thin focal plane and is only marginally influenced by objects outside the focal plane [7]. DIC also furnishes the ability to distinguish between two regions of differing optical thickness, allowing the clinician to distinguish internal structures such as vacuoles and acrosomes, which are invisible in bright-field images due to their being as transparent as their surroundings.





illumination beam passes through a slit aperture. Following this, the light is deflected by regions in the sample of increasing or decreasing optical thickness. This deflected light is then modulated to be darker or lighter based on the deflection. This results in producing contrast that is determined by the position and slope of the phase gradients in the sample [3].

Some of the advantages of HMC are the generation of DIC-like images with relatively low cost of the accessories required. HMC is also not affected by the presence of birefringent materials (such as a plastic dish containing the sample) as is DIC, as the sample is not placed between two polarizers. However, HMC cannot generate the high level of detail needed for intracytoplasmic morphologically selected sperm injection as the placement of a slit before the condenser prevents the full NA of the condenser from being utilized, resulting in a decrease in resolution and a corresponding decrease in the ability to discriminate between small details in the sample [3]. As in DIC, no quantitative data is obtained regarding the 3D structure of the sample.



Intracytoplasmic morphologically selected sperm injection (IMSI)

IMSI is a relatively new and popular imaging method used in sperm selection for intracytoplasmic sperm injection (ICSI). IMSI makes use of the DIC method to receive high detail, high contrast images of sperm cells. The DIC image is captured using a 60x or 100x oil objective and subsequently enhanced through use of a magnification changer, video-zoom and digital camera and imaging system [3,8]. The total magnification is then derived as follows:

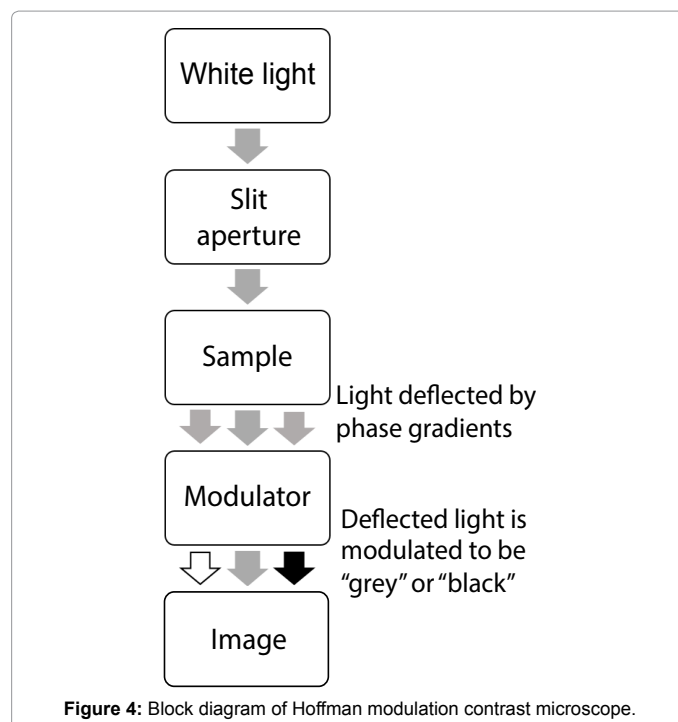
$$\text{Total magnification} = (\text{magnification of objective lens}) \times (\text{magnification of magnification changer}) \times (\text{magnification of the video-zoom}) \times (\text{diagonal length of final display screen} / \text{diagonal length of CCD of camera})$$

Typically magnifications of 6000x or greater can be achieved; however, it is important to understand that the actual resolution of the image cannot be improved by using this method as the resolution

While DIC is a great improvement over bright-field in terms of contrast and the ability to distinguish internal structures, it is important to realize its inherent limitations. DIC does not provide quantitative data as to the 3D structure of the sample; it simply produces a pseudo-3D image, making cell structure more visible to the observer. However, this image does not have real 3D meaning, and a computational 3D analysis of sperm cells based on DIC images is not possible. Furthermore, while in DIC the edges of regions in the sample are made visible, the internal regions themselves all share gray-levels similar to that of the background; thus not all points on the image have meaningful value. Figure 1(C) shows the same sperm cell imaged in Figures 1(A) and 1(B), but now imaged with DIC microscopy. As can be seen, in this image the cell appears to be 3D-like to the observer, standing up out of the image, with edges on the right side of the cell being brighter while edges on the left side of the cell appear darker. The acrosome appears to be flatter than the nucleus, which appears to be taller, allowing the observer to distinguish between these two regions.

Hoffman modulation contrast (HMC)

HMC is a phase contrast method that produces a pseudo-3D image similar in appearance to that of DIC, but does so by employing a different optical mechanism. As shown in Figure 4, in HMC the



is determined by the NAs of the microscope condenser and objective lenses [3]. Thus, we cannot expect to discriminate between details smaller than the ones provided by the standard DIC optical system. Almost all magnification following the objective, digital or otherwise, is simply “empty magnification”, the image has been made to appear larger to enable easier visualization by the observer, but it has not been improved in terms of resolution or detail and no new information is provided; thus this empty magnification does not provide any advantage for computational analysis.

Currently there is some controversy as to whether IMSI is effective in improving healthy fertilization rates. Numerous studies have attempted to compare standard ICSI to IMSI with varying results. Several studies show improvement in healthy fertilizations when implementing IMSI, especially for couples who have undergone and experienced repeated ICSI failure [9-12], while other studies indicate that IMSI does not produce any significant increase in successful fertilizations and implantations [13,14].

Polarized light microscopy (PLM)

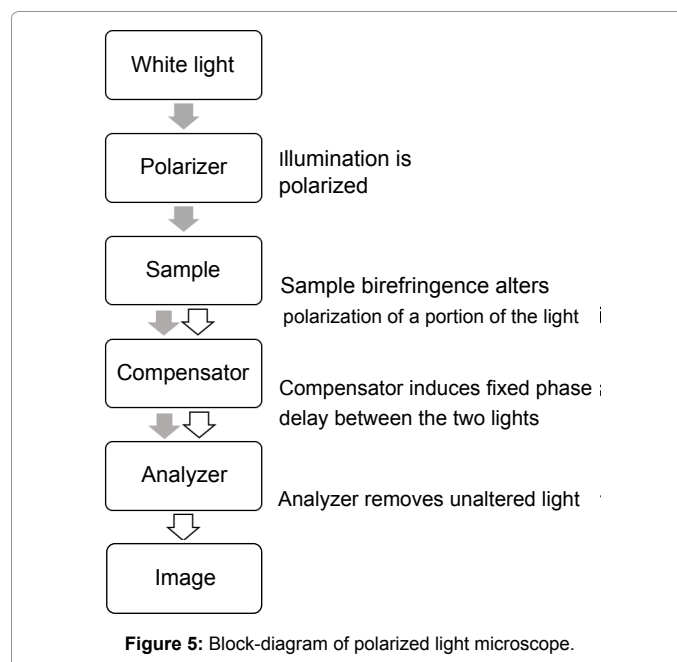
PLM is a method that is seeing increased use in evaluating sperm and especially ova for IVF and ICSI. This method takes advantage of the birefringence property possessed by materials composed of well-ordered molecular structures and polarizable bonds. When polarized light passes through a birefringent material, such as the head of a sperm, the material acts as a polarizer, shifting the polarization angle of the light. A phase delay is induced as well. By polarizing the illumination beam prior to the sample and then passing the light from the sample through another polarizer, at a 90° angle orientation to the first polarizer, we will only see light from the birefringent regions of the sample, as only there does the light undergo polarization changes. Thus, in the resulting image, the birefringent regions are brighter than other regions and the background is completely dark. A block diagram of this system is shown in Figure 5 [15].

PLM may also be used to produce quantitative data regarding the phase delay induced by the birefringence. This data can then be used to find the refractive index or the thickness of the birefringent sample. In order to perform quantitative PLM a compensator is placed between the objective and the analyzer. This compensator induces a fixed phase delay between the portion of the light that was polarization shifted when traversing the sample relative to the non-shifted light. This allows interference between the two lights to occur in a controlled manner, producing an image where intensity is dependent on the phase delay induced by the birefringence [15].

Studies show that measurement of sperm head birefringence may help determine whether sperm are in possession of functioning acrosomes, as well as indicating the level of order and compaction present in the acrosome and nucleus [16]. The level of sperm head birefringence may also indicate whether the DNA is fragmented and damaged [17]. The presence and level of birefringence in sperm heads may also indicate sperms that are more likely to result in implantation and pregnancy, especially in IVF where severe male factor conditions are involved [18-20].

Interferometric phase microscopy (IPM)

Interferometric phase microscopy, also known as holographic microscopy, may be the next stage of evolution for imaging in IVF. In contrast to other phase imaging methods, holographic imaging provides the possibility to obtain quantitative images, meaning images that possess meaningful values at all of their points. In these images or phase maps, the value at each point represents the optical thickness



of the sample, which is defined as the product of the sample thickness and its refractive index. While in DIC interference is created between two intermediate images that are identical and laterally shifted, in holographic imaging the second image is created by a reference beam, a beam that did not traverse the sample, and is an image of uniform phase. Overlaying these two images produces an interference image as in DIC, yet with very different results. Due to the uniform phase of the reference beam, we receive an image filled with alternating bright and dark lines, known as interference fringes. Using computer algorithms, these fringes can be extracted and processed in order to generate a phase map in which the value of each pixel is the actual optical thickness or optical path delay (OPD) of the sample. In this manner, we receive quantitative data regarding the 3D structure of the sample as well as the 2D position of internal structures based on their refractive index [21]. Figures 1(D) and 1(E) show the same sperm cell imaged in Figures 1(A)-1(C), but now imaged with holographic microscopy. As can be seen in these images, the edges of the cell are clearly visible and the acrosome can be easily distinguished from the nucleus. As well, the value of each pixel is equal to the OPD measured in nanometers, as indicated by the color bar.

There exist significant advantages to holographic imaging of sperm for IVF. First and foremost, it has been shown that assessment of sperm according to the World Health Organization criteria for sperm selection [22] by holographic imaging garners results correlating to those produced by label-based bright-field microscopy [23]. In addition, holographic imaging generates quantitative data regarding the thickness of the cell and provides the clinician with data regarding the 3D structure of the cell. The acrosome and nucleus can easily be identified and measured as in labelled sperm, as the acrosome has a significantly lower OPD due to its lesser thickness. Vacuoles can be located and measured due to their decreased refractive index, resulting in a drop in OPD where vacuoles are situated. Furthermore, new parameters are becoming available through holography. Dry mass can be calculated based on the OPD, enabling analysis of the actual amount of biomass contained in the whole sperm or select regions of the OPD image, such as the nucleus [21]. This may lead to the development of new metrics for sperm assessment in the near future. One more

significant advantage to holographic imaging is the possibility to numerically refocus the acquired holograms, a feat made possible by the capturing of data detailing the complex wave as opposed to only the intensity [24].

As with any new technology, there are limitations and drawbacks. In most cases holographic imaging is made possible through use of coherent illumination such as a laser. Performing holographic imaging with a highly coherent (single color) source causes holograms to be noisy due to speckling noise. As well, the need for a reference beam that travels a different optical path than that of the sample beam leads to fluctuations and noise caused by vibration and shifting air currents in one or both paths. Holographic imaging systems generally need to be situated on vibration isolation tables as even the smallest of vibrations causes fringes to shift and blur. Furthermore, until recently, these systems were expensive “open microscopes” that required optical expertise in order to be aligned and operated.

Novel Clinical Applications

The advent of holographic imaging and the quantitative 3D data it provides enables new measurements and applications, aside from those already detailed above.

In 2012, Su et al. implemented a dynamic high-throughput method whereby multiple live sperm can be tracked in 3D space and their 3D trajectories recorded and analyzed. The system used was lens-free, with sperm being imaged on a chip and being illuminated by a blue LED and red LED simultaneously and from different angles. Based on the two holograms extracted from the red and blue light respectively the 3D trajectories of the sperm were analyzed, enabling accurate measurement of parameters such as curvilinear velocity, straight-line velocity, etc. [25,26].

In 2014, Di Caprio et al. developed a method for automated detection and tracking of sperm using a microscope with a fixed objective-sample distance. This method produced results comparable to those achieved by Su et al., albeit through use of holographic imaging utilizing a single light source, as well as the numerical refocusing technique mentioned above in order to determine the sperm z-position [26,27].

In 2013, Merola et al. published their findings on a fast, high-throughput and label-free system for estimating the biovolume of sperm cells quickly and simply. Their system utilizes optical tweezers to trap and rotate the cells as they flow through a microchannel, enabling holographic imaging of the sperm from multiple angles and the production of a tomographic 3D model. Aside from enabling high-throughput, imaging the live sperm in a microchannel allows the sperm to retain its natural shape, as opposed to the altered shapes produced when sperm adheres to a slide or is crushed under a coverslip [26,28].

Gap to Clinical Use

At this time, there is little preventing holographic imaging from being utilized in IVF clinics, as certain commercially available holographic imaging microscopes are already seeing clinical use. The main obstacles that need to be overcome are expense and training, as well as further studies. With regard to expense, holographic imaging systems are precise equipment, more so than many microscopy systems currently in clinical use. In a holographic imaging system that is sensitive to OPDs on the scale of nanometers, the precision required in construction of such a device may lead to holographic microscopes being significantly more expensive than the other systems detailed above. As holographic imaging systems are so sensitive, they require vibration isolation in order to prevent small vibrations transmitted

by the table from affecting measurements. Clinics may be unwilling to expend significant resources on a holographic imaging microscope when no clinician present is trained in its use, and while few clinical trials have been performed to prove its value.

A further difficulty lies in training clinicians in the use of holographic imaging and the interpretation of the OPD images. Work is currently ongoing on computer assisted diagnosis algorithms that will interpret the 3D data and provide the clinician with recommendations, with the aim of enabling automated sperm selection at high throughput. Further studies regarding the effectiveness of sperm selection through holographic imaging will likely be required before many IVF clinics will be convinced of the value of holographic imaging.

Compact Interferometric Modules (CIM)

A possible solution for defraying the expense of acquiring new holographic microscopes, one that may allow holography to be introduced to clinics sooner, lies in the use of CIM. These modules, such as the TAU interferometer [29], are capable of adapting existing microscopes for use as holographic microscopes.

The TAU interferometer can be placed at the camera port of a microscope illuminated by a weak laser beam and is capable of producing holograms by splitting the inputted sample beam into two beams, cleaning all sample information from one beam in order to produce a reference beam and then recombining the beams to generate a hologram. The unit itself is a small box and contains very few optical elements, making production of this unit very cost-effective [29].

Other CIM have been developed as well, such as the flipping interferometry unit proposed by Roitshtain et al. [30]. This unit, as well as other units [31,32] produces holograms by means of interfering the sample beam with itself by overlaying an empty section of the image over the sample region of the image. This method is simple and effective, so long as it can be arranged that part of the image remains sample-free and the correct illumination is used.

Closing Remarks

Holographic imaging promises significant advancements in the field of sperm analysis for IVF. The 3D structural information as well as dry mass values provided by holographic imaging supplies the clinician with more quantitative data upon which to select sperm. The improved contrast of holographic images is also significant, allowing clinicians to view the sperm with contrast similar to that of labelled sperm and to more accurately determine sperm morphology. As well, 3D tracking of sperm will enable clinicians to more accurately and objectively assess sperm motility. While current imaging methods have been sufficient for decades, with the introduction of simple and portable holographic modules, holographic imaging is now poised to bring the field of sperm selection to its next stage of development.

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