Analysis of Bovine Sperm Cells by a Combined Holographic and Raman Microscopy Approach

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Abstract—In this work, an approach based on digital holography (DH) combined with Raman spectroscopy (RS) is used for a complete label-free analysis of single bovine spermatozoa. DH allows to obtain high-resolution images of bovine sperm from the reconstruction of a single acquired hologram, highlighting in some cases morphological alterations. Quantitative 3D reconstructions of sperm head, both normal and anomalous, have been studied and an unexpected structure of the post-acrosomal region of the head has been detected. DH microscopy analysis have also confirmed such anomalies, suggesting the protein vibrations as associated Raman marker of the defect.

Keywords—spermatozoa; biophotonics; digital holography microscopy; Raman spectroscopy; Raman imaging

I. INTRODUCTION

Considering the growing interest of cattle producers in identifying the most healthy and fertile bulls, several methods have been suggested for assessing sperm quality, with particular care to sperm with intact acrosomes, sperm with normal morphology [1-3] and sex sorting [4]. An effective semen analysis should be able to detect the spermatozoa morphological and biochemical alterations, without affecting the spermatozoa viability. Consequently, the used techniques have to be: i) label-free, to reduce costs and exclude all unwanted effects that may be introduced by labels; ii) non-destructive, to avoid any vitality alteration of the analyzed sperm; iii) independent from the experience of the technician and environmental conditions. Thus, photonic methods could represent very promising tools [5]. Among the optical techniques useful for the morphological characterization of the semen, optical microscopy and AFM represent unsuitable solutions: time-consuming, the first; difficult and expensive, the second [6,7].

In this work, we propose a combined Digital Holography (DH) [5] and Raman Spectroscopy (RS) approach [4] as powerful techniques for a non-destructive, morphological and biochemical characterization of the spermatozoa.

Since the 1970s, holographic techniques have been applied in the interdisciplinary fields of biomedicine and life sciences [8] as well as in new research areas such as biophotonics [9]. DH microscopy opens up new perspectives for biomedical applications, allowing the visualization and detection of displacements and movements and considering that this technique can be applied nondestructively, marker free, full field (no scanning required), and online (video repetition rate) [10]. Moreover, DH microscopy allows the reconstruction of 3D maps of single selected cells and measurements of cell volumes/thickness [11-14]. Finally, DH gives the advantage of directly observing spermatozoa in their native environment even if the sperm cell appears almost transparent by using a bright field microscope, since its optical proprieties differ slightly from the surrounding liquid. Indeed, the light beam that passes through a spermatozoon undergoes a phase change, in comparison to the surrounding medium, whose value depends on the light source, the thickness and the integral refractive index of the object itself. This phase-contrast can be measured with high-resolution by DH microscopy [5].

When the morphological analysis needs to be integrated with biochemical information, RS can be efficiently used. Indeed, RS is a sensitive and non-invasive method with resolution in the sub-micrometer range that allows the chemical assessment of single cells by detecting their biomolecule vibrations, serving as a cellular intrinsic ‘fingerprint’ [4, 15-23]. This technique offers a huge potential for solving biomedical problems, such as single-cell characterization [24], biomarkers detection [25] and sex-identification for instance [4]. Additionally, RS is more suitable for studies of aqueous samples, compared to infrared spectroscopy, as water has very weak Raman scattering properties. It is particularly suitable for in vivo measurements, because the laser powers and excitation wavelengths that are used do not affect the biological sample [26]. Furthermore, it does not require any label or marker, as in fluorescence microscopy for instance, allowing the rapid and non-invasive analysis of the samples in situ [27]. RS can be also used to provide pseudo-color images according to the Raman spectral band intensities allowing the identification of cell phenotype and physiological state [21-23, 28-32].

In this study, DH and RS are used for a complementary morphological and biochemical bovine sperm cell analysis [23].

II. MATERIALS AND METHODS

II.1 Theoretical background

DH is a technique based on interference between two laser beams commonly named reference beam and object beam, the latter illuminates the object under test. Owing to their different optical path, both waves interfere at the surface of the recording device, and the hologram is proportional to the intensity of this interference pattern. The object (in our experiment is a...
biological object) scatter incoming light, forming the complex object wavefield:
\[ O(x, y) = |O(x, y)|e^{i\phi(x,y)} \]  
(1)
where \(|O|\) is the amplitude and \(\phi\) is the phase, \(x\) and \(y\) correspond to the Cartesian coordinates in the recording plane (hologram plane). Intensity and phase distributions can be retrieved starting from the complex object wavefield according to the following relations [33]:
\[ I(x, y) = |O(x, y)|^2 \]  
(2);
\[ \phi(x, y) = \text{arctan} \left( \frac{\text{Im}[O(x,y)]}{\text{Re}[O(x,y)]} \right) \]  
(3).

Since the phase distribution is obtained by a numerical evaluation of the \(\text{arctan}\) function, the values of the reconstructed phase are restricted in the interval \([-\pi, \pi]\), i.e., the phase distribution is wrapped into this range. In order to resolve possible ambiguities arising from thickness differences greater than \(\lambda/2\), phase-unwrapping methods have to be generally applied.

Quantitative information about the thickness of the sample under test can be obtained by considering that the phase \(\phi(x, y)\) is related to the optical path difference (\(\text{OPD}\)) through the equation:
\[ \Delta \phi(x, y) = \frac{2\pi}{\lambda} \text{OPD} \]  
(4)
where a transmission configuration has been considered. The \(\text{OPD}\) depends on the refractive index and thickness both of the biological sample and the material containing the object itself [9]. The phase of the object wavefield is encoded in the intensity fringe pattern adding the reference beam \(R(x,y)=|R(x,y)|\).

The hologram is acquired by an image sensor (CCD or CMOS); this corresponds to a 2D rectangular raster of \(M \times N\) pixels, with pixel pitches \(\Delta x\) and \(\Delta y\) in the two directions [11, 34]. The complex field of the object beam is then obtained by applying a mathematical analysis [33]. In particular, the hologram is 2D Fourier transformed. A small angle has been introduced between the two interfering beams in our setup (off-axis configuration) to spatially separate the first-order diffraction from the whole spatial frequency spectrum. Thus, in order to retrieve the full-field distribution of the object beam, the first-order diffraction is bandwidth filtered and shifted to the origin of the k-space [35]. By knowing the whole field, the optical wavefront can be reconstructed at different distances from the plane of acquisition applying the Fourier formulation of the Fresnel-Kirchhoff diffraction formula [36].

Each value of the matrix forming the DH microscopy phase map represents the phase delay of the light passing through the sample, which is connected with the thickness of the cell. This kind of information is purely quantitative and can be simply used for the morphological analysis of the cells and for the selection of the anomalies.

RS is a light scattering technique commonly used to provide a chemical “fingerprint” by which molecules can be identified. When a monochromatic radiation impinges on a sample most photons are elastically scattered (Reyleigh scattering), therefore they have the same energy as the incident photons. Only a small fraction (approximately 1 in 1 million) is inelastically scattered at optical frequency different from the frequency of the incident photons. Photons of the laser light are absorbed by the sample and then they are reemitted at frequency shifted up or down in comparison with original monochromatic frequency, which is called the Raman effect. This shift provides information about vibrational, rotational and other low frequency transitions in molecules. In particular, the difference in energy between the incident and the scattered photon, or Raman shift, corresponds to the energy of a vibration of the scattering molecule:
\[ \bar{\nu} = \frac{1}{\lambda_{\text{incident}}} - \frac{1}{\lambda_{\text{scattered}}} \]  
(5)

The origin of the modified frequencies found in Raman scattering is explained in terms of energy transfer between the scattering system and the incident radiation. Therefore, a Raman band is to be characterized not by its absolute wavenumber but by the magnitude of its wavenumber shift \(\bar{\nu}\). A plot of intensity of scattered light versus energy difference is a Raman spectrum. Raman scattering can occur with a change in vibrational and rotational energy of the molecule. Since the energy levels are unique for each molecule, a Raman spectrum provides a “fingerprint” of the sample, allowing identification of unknown sample components with a precision that is unmatched by other techniques [21,22,37-39].

Raman spectroscopy can be used to study solid, liquid and gaseous samples. Additionally, by combining RS with a microscope it is possible to reconstruct the distribution of assigned chemicals within a cell. This kind of application is usually referred as Raman imaging or mapping [20,32,40,41]. Raman image can be generated by raster scanning the sample through the laser probe and collecting a spectrum at each position. Finally, the collected data can be processed to generate false color images based on the sample biochemical composition [21, 39].

II.2 Experimental set up

A scheme of the DH system used in this work is shown in our previous paper [5,23]. Briefly, the light source was a 633 nm He-Ne laser. The laser beam, filtered and expanded, was divided into reference and object beam by a pellicle splitter. The object beam was sent to the sample and collected through a microscope objective (40x, 0.65 N.A.). Then, reference and object beams were recombined by a cube beam splitter. Finally, the generated holographic pattern was projected onto a CCD camera (1392 x 1040 pixels array; area of a single pixel 4.7 x 4.7 mm²).

Raman images were acquired by using a commercially available inverted Raman microscope (XploRA Horiba, Jobin Yvon) with a 532 nm laser excitation. The Raman spectra were measured using a 100x magnification objective (Nikon, 1.1 N.A.) in back-scattering geometry. The monochromator was equipped with a 600 lines/mm holographic grating and the spectral resolution achieved was about 2 cm⁻¹. The Raman signals were detected using a back-illuminated CCD camera, thermoelectrically cooled.

II.3 Sample preparation
The bovine sperm cells to be analyzed were prepared by the Institute “Lazzaro Spallanzani” after fixation in suspension of the seminal material with 0.2% glutaraldehyde solution in phosphate buffered saline (PBS) without calcium and magnesium (1:3 v/v) 3. A 4-μL solution drop has been deposited in the sample chamber. The sample chamber used in our experiments was made by putting an 80-μm deep vinyl spacer between a quartz slide (1 mm in thickness) and a quartz coverslip (150 μm in thickness) (UQG). The cover slip has been linked to the glass slide by means of a strip of varnish. The cells were allowed to sediment (for 30 min) on the quartz coverslip before starting the experiments.

III. RESULTS AND DISCUSSION

DH microscopy approach was used to investigate the morphology and structure integrity of the bull spermatozoa [28]. The hologram was acquired using a double exposure technique. The first exposure was made on the sample under investigation, while the second one was made on a reference surface in proximity of the object. Information about the aberrations caused by the optical components, like, for example, the defocusing due to the microscope objective, was incorporated into the second hologram. In such a way, after data acquisition, these aberrations can be compensated by numerically processing the two holograms [11]. The reconstructed three-dimensional shape of a bovine spermatozoon is reported in Fig. 1(a). Since the phase \( \phi(x, y) \) is known, quantitative information about the thickness of the sample can be obtained by Eq. (4). Figure 1(b) shows an interesting feature, common to many (about 2-3% of the total cell number) analyzed spermatozoa: a “protuberance” on the post-acrosomal region of the head. This alteration is well visible in the profile reported in figure 1(b) relative to the line LL’ shown in Fig. 1(c) [13,23]. A first explanation for the estimated area and volume variation is that the “protuberance” highlighted by DH microscopy could be due to the presence of the centrioles into the structures connecting the tail to the head [23]. Among possible explanations of the “protuberance” nature, an artifact due to a possible different refractive index between the post-acrosomal region, (containing almost exclusively highly compact chromatin) and the acrosome should be excluded. Thus, the same characteristic has been observed in images acquired by dual core confocal 3-D Leica microscope (DCM), reported in our previous paper [13], excluding the artifact hypothesis. The results presented in Fig. 1 clearly highlight the main advantage of the holographic approach: it provides a 3D reconstruction of the spermatozoon from a single acquired image (i.e., the hologram), without any mechanical scanning. However, both holographic and confocal images only reveal the presence of morphological defect, while they are not able to identify its biochemical constitution.

Complementary information on the biochemical structure of spermatozoa can be achieved by Raman imaging. All the acquired Raman spectra were corrected for the quartz coverslip and PBS solution Raman spectrum, by subtracting the background spectrum. The spectra were base-line corrected using five order polynomials and thereafter, normalized to the maximum Raman peak. All the spectra were calibrated by acquiring the Raman spectrum of a polystyrene bead.

In figure 2(a), we reported three spectra, acquired in three distinct regions of the spermatozoa: the tail (blue line), the nucleus (green line) and the acrosome (magenta line). The corresponding Raman band assignment is summarized in Table 1 [4]. The nucleus Raman spectrum (green line) is characterized by strong Raman bands corresponding to the nucleic acids and DNA (726, 785, 1095 and 1581 cm\(^{-1}\)). Pronounced bands associated with the proteins and lipids (1200–1300, 1480 and 1600–1680 cm\(^{-1}\)) identify the acrosomal vesicle spectrum (magenta line). Finally, the tail Raman spectrum (blue line) is characterized by a sharp peak around 751 cm\(^{-1}\) previously assigned to mitochondria and strong peaks at 1005 and 1450 cm\(^{-1}\) consistent with the presence of proteins in this spectral region.
By assigning a specific color to each spectrum, the false color image in figure 2(b) can be reconstructed. The spermatozoon Raman image has been obtained by scanning the sample under microscope with a step of 0.2 µm over a total mapping size of 9 x 12 µm². The Raman excitation power on the sample was about 15 mW. Spectra were recorded at each pixel with an integration time of 10s. As for the DH, the Raman image reveals the “protuberance” on the post-acrosomal region of the head. The protuberance can be better identified from the 2D intensity map reported in figure 2(c), clearly showing a local concentration of material in the sperm region connecting the tail to the head.

A detailed inspection into figure 2(a) (blue line) demonstrated that the sharp vibrations originating from the presence of proteins in the spectral region at 1005 and 1450 cm⁻¹, clearly less intense in the other spectra, together with the presence of mitochondria, correspond to the most important Raman markers of such “protuberance”. Our results suggest an intriguing correlation between the amount of protein and the presence of the “protuberance” that could be associated with the presence of centrioles in the sperm region connecting the tail to the head. Indeed, the centrioles are cylindrical cell structures composed essentially by tubulin, therefore affecting the local concentration of protein. However, in order to give a correct biological explanation to the origin of this formation, additional analyses and comparisons with traditional fluorescence confocal microscopy results are required.

IV. CONCLUSIONS

A Digital Holography-Raman microscopy combined approach was used in this study for a label-free, morphological and biochemical characterization of bovine sperm cells. From the reconstruction of a single acquired hologram, high-resolution images of the sample have been obtained, clearly highlighting some morphological alterations. In particular, in the post-acrosomal region of the head we have identified an anomalous “protuberance” of the head. Raman imaging has also been performed in order to study the biochemical structure of the sample. The measured Raman images have confirmed the presence of the “protuberance” in the spermatozoon head, suggesting a correlation with the increase of protein concentration (probably due to the presence of centrioles) in the region connecting the tail to the head. The current study sets the foundation for further investigations on the sperm quality assessment both in animals and in humans. In particular, we are implementing around the same optical microscope a novel combined system allowing the simultaneous holographic and biochemical characterization of the sample [23]. Alternative experimental configurations, such as the implementation in microfluidic environments [28], the adoption of compressive methods [20,42] and improving data analysis [36], are required in order to improve the DH-RS approach throughput, in terms of automation, higher analyzed cell number and sensitivity/specificity.

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