The Talbot effect in self-assembled red blood cells investigated by digital holography

Pasquale Memmolo, Lisa Miccio, Francesco Merola and Pietro Ferraro
CNR-ISASI, Institute of Applied Sciences and Intelligent Systems, ‘E. Caianiello’, Via Campi Flegrei 34, 80078, Pozzuoli, Napoli, Italy
E-mail: pasquale.memmolo@isasi.cnr.it

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Abstract
The Talbot effect, also known as self-imaging, is a well-established phenomenon observed when a beam of light is transmitted through a periodic pattern and the image of the pattern is reproduced at a regular interval along the optical axis, namely the Talbot length. This effect has been widely investigated and exploited for several applications in different fields. Here we discuss for the first time, to the best of our knowledge, the self-imaging effect due to a self-assembled and quasi-ordered array of live biological cells under illumination by a coherent light beam. In particular, self-assembly of red blood cells (RBCs) provides a monolayer of cells that appear to be quasi-ordered in a trigonal array geometry. Thanks to the recent proof that RBCs can be modeled as a microlens array, the Talbot length can be predicted and the corresponding self-imaging can be observed experimentally. In particular, we investigate the Talbot effect of self-assembled RBC arrays for two different RBC body shapes, i.e. discocytes and spherocytes, by using digital holography as tool for imaging and quantifying this phenomenon. This research could open up a new way to investigate biological material by exploiting its photonics properties.

1. Introduction
Self-imaging is a widely explored phenomenon related to the transverse periodicity of a diffraction pattern illuminated by a beam of light. The theory of wave propagation ensures the appearance of strict periodicity along the propagation axis at certain regular intervals. This amazing effect has been investigated for over a century since the discovery by Talbot of self-imaging of periodic objects illuminated by very small white light sources [1]. Since the 1960s, the so-called ‘Talbot effect’ has attracted many researchers who have exploited this phenomenon to find solutions to various scientific and technological problems in optics [2–4]. In fact, the Talbot effect has been used for the measurement of the refractive index of transparent [5] and birefringent materials [6], to measure the displacement of a deformed object [7] or its roughness [8], for lithography [9, 10], for imaging [11, 12] and for spectrometry by multilambda digital holography (DH) [13]. In particular, for the characterization of the foci of microlens arrays, in [14] the authors demonstrate that the foci of a finite two-dimensional (2D) periodic microlens array also form a periodic structure, thus exactly reproducing foci in the Talbot planes but also showing reproduction of multiple foci in the fractional Talbot planes [15]. The fractional Talbot effect consists of more complex weighted replicas of the original object and can be used to replicate images of non-periodic objects by means of Talbot array illuminators [16]. A generalization of this self-imaging, named the nonlinear Talbot effect [17], focuses on the formation of second-harmonic self-imaging of the generated periodic intensity pattern at the output surface of a crystal.

The periodicity of the array seems to be the fundamental prerequisite for achieving self-imaging at the Talbot length, as well as fractional ones. Recently, self-imaging of a quasi-periodic grating has also been demonstrated [18], thus opening the way for the investigation of more complex array structures. Finally, it is important to note that 2D periodicity of a structure or microlens array can be obtained by considering different arrangements with respect to the classical squared structure, i.e. arrays with diagonal and hexagonal symmetry [19–21]. In such arrangements the self-imaging distance differs from the Talbot length as it is usually defined. A second aspect to be considered is the microlens shape, which is spherical in all the
induced mechanical stress by optical tweezers. Length changes with respect to the squared case and the spatial period of an array with hexagonal symmetry is shape. We show that the Talbot effect in self-assembled quasi-hexagonal RBC structures can be predicted and (ii) where the medium is a hypotonic solution, thus provoking the swelling of the cells to a spherical-like experiments: (i) in which a heathy discocyte RBC sample is immersed in its physiological isotonic solution simulating the propagation of a plane wave through these patterns. Actually, we consider two different images (QPIs). Then, we model these QPIs as microlens arrays and the Talbot length is determined by reconstruction of the recorded digital holograms of RBCs with the aim of retrieving the quantitative phase aberrations. In all previous applications the optical behavior of one biological cell at a time was considered. Here for the first time we show the cooperative effect as a microlens array of biological cells. In our investigation we can consider a self-assembled RBC array effectively as a microlens array, and employ DH microscopy [32] as the tool for imaging the sample and to determine the Talbot length. It is interesting to note that self-assembly of RBC-like colloidal particles with the same shapes and sizes was used to create monolayers with perfect trigonal symmetry [33]. In our experiments we observe that RBCs tend to arrange themselves naturally with a quasi-periodic trigonal (or hexagonal) symmetry, according to the ideal case. We perform numerical reconstruction of the recorded digital holograms of RBCs with the aim of retrieving the quantitative phase images (QPIs). Then, we model these QPIs as microlens arrays and the Talbot length is determined by simulating the propagation of a plane wave through these patterns. Actually, we consider two different experiments: (i) in which a healthy discocyte RBC sample is immersed in its physiological isotonic solution and (ii) where the medium is a hypertonic solution, thus provoking the swelling of the cells to a spherical-like shape. We show that the Talbot effect in self-assembled quasi-hexagonal RBC structures can be predicted and experimentally proved. We compare the experimental results with simulations.

2. Materials and methods

2.1. Basic theory of the Talbot effect and simulations

The theory of the Talbot effect is based upon classical diffraction theory [3] and has been developed over decades for explaining the phenomenon and applying it to many situations. In this paper we just consider the basic mathematical formulation needed for the general reader to easily understand the Talbot effect. The self-imaging is generally observed when a beam of light is transmitted through a periodic pattern and a replica of the pattern is reproduced at integer multiples of a specific position on the optical axis, namely the Talbot length.

Since in our investigation we are considering 2D RBC arrays as pure phase microlenses, we focus our discussion on the calculation of 2D Talbot lengths for squared and hexagonal periodic patterns. In figure 1 we show a schematic view of the Talbot effect for a squared pattern.

If one considers monochromatic light incoming to a squared array of microlenses and observes the propagation of light through the array, at a certain distance along the optical z-axis all diffraction orders are in phase and reinforced if the following condition is satisfied [4]:

\[ z = 2k \frac{d_s^2}{\lambda} = 2kZ_T \]  

(1)

where \( k \) is a positive integer, namely the self-imaging number, \( d_s \) is the spatial period (which is the same along vertical and horizontal axes due the squared symmetry) and \( \lambda \) is the wavelength of the incoming plane wave. Therefore, by fixing \( k = 1 \), the primary Talbot length is defined as \( 2Z_T \) while \( Z_T \) is named the secondary Talbot length [4]. An example of a squared array of pure phase spherical lenses is shown in figure 2(a), in which \( d_s = d_x = d_y \). In this simulation, \( d_s = 10.5 \mu m \) and \( \lambda = 0.532 \mu m \), thus corresponding to the primary Talbot length \( 2Z_T \approx 414.5 \mu m \).

The case of a microlens array with hexagonal symmetry has been investigated due to its spontaneous arrangement, as shown in figure 2(c). For such a geometric pattern, which has trigonal symmetry, the Talbot length changes with respect the squared case and the spatial period of an array with hexagonal symmetry is...
Figure 1. Schematic view of the Talbot effect for a squared pure phase microlens array.

Figure 2. Simulation of squared (a) and hexagonal (c) spherical arrays of microlenses and identification of the corresponding Talbot length through the Tamura coefficient (b), (d). The inset figures in (b) and (d) show the intensity patterns obtained at primary (orange), secondary (green) and fractional (violet) Talbot planes which correspond to some peaks of the Tamura coefficient.

defined by the distance between each microlens and the neighboring ones, assembled in a hexagon. Let this period, $d_H$, be defined as

$$z = k \frac{3d_H^2}{2\lambda} = kZ_T. \quad (2)$$
Notice that the trigonal arrangement is guaranteed if the form factor of the hexagon is such that \( d_f = \sqrt{3} d_l \) and \( d_s = d_l \). By using the simulated values \( d_l = 10.5 \) and \( \lambda = 0.532 \, \mu m \), the Talbot length is \( Z_T \approx 310.75 \, \mu m \). Finally, the fractional Talbot effect is observed at all rational multiples of \( Z_T \), namely \( Z_f = (p/q)Z_T \), where \( p \) and \( q \) are co-prime integers. The corresponding images consist of \( q \) equally spaced replicas of the transmission function of the phase microlens array.

It is important to note that each image at multiples of the Talbot length, whether it is primary, secondary or fractional, provides a higher local image contrast. Therefore, in order to evaluate the contrast of the reconstructed wavefront intensity, a suitable metric can be used to identify the Talbot length, varying the reconstruction distance. To this end we employ the Tamura coefficient (TC), which has been successfully used as a refocusing metric for holographic reconstructions [32]. Let \( I_z \) be the intensity of the wavefront reconstructed at distance \( z \), then \( TC_z = \sigma(I_z)/\mu(I_z) \) where \( \sigma \) and \( \mu \) are the standard deviation and the average operators, respectively. In figures 2(b) and (d) we report the TC for a wavefront propagated numerically up to 500 \( \mu m \) from the microlens array plane for the two cases simulated in figures 2(a) and (c), respectively. The illumination wave plane was considered with \( \lambda = 0.532 \, \mu m \). In particular, by varying the reconstruction distance for the squared pattern (see figure 2(b)) and the hexagonal (see figure 2(d)) microlens arrays, the TC trends provide local maximum peaks corresponding to the main and fractional Talbot lengths (see inset boxes). In the orange insets within figures 2(b) and (d), the focal planes and the corresponding self-images, obtained at the predicted Talbot lengths, are shown along with the secondary Talbot images (in green). Finally, two fractional Talbot images are highlighted in violet boxes, corresponding to Talbot lengths \( Z_T/2 \) and \( Z_T/3 \) for the squared and hexagonal cases, respectively. Theoretically, for a microlens array with an exact period, self-imaging can be observed at any predicted length, independently of the shape of the lens. However, the longer the Talbot length the greater the degradation in the self-image. This degradation is mainly caused by the divergence of the diffraction in the far field, since the Talbot effect is a near-field diffraction phenomenon. In figures 2(b) and (d) this behavior can be clearly observed by noting that the image contrast in the Talbot planes is gradually lost as the Talbot length increases (see the plots of the Tamura coefficient).

2.2. Sample preparation and holographic processing
The possibility of obtaining self-imaging of naturally assembled RBC arrays was investigated for two blood samples, i.e. healthy discocyte RBCs and spherical-like RBCs obtained by managing the osmolarity of the medium. The blood samples were collected from a healthy volunteer who gave informed consent according to European ethics guidelines. The experiments were conducted with full respect for the European Charter of Fundamental Rights (2000/C 364/01, Article 3, Article 8). Heparinized blood drawn within the hour before use was employed for the experiments. The sample was prepared by the following protocol. About 3 ml of heparinized whole blood was withdrawn into a hemocrit tube. Blood was centrifuged at room temperature for 15 min at 2500 r.p.m. to separate it into its parts, i.e. plasma, buffy coat and RBCs. Then, the pellet was collected and about 100 \( \mu l \) of erythrocytes was diluted with a saline solution of 0.9% w/v of sodium chloride in sterile water to obtain a final sample volume of 2 ml. The osmolarity of the medium in the natural isotonic condition was about 308 mOsm \(^{-1}\). By reducing the medium osmolarity to about 205 Osm \(^{-1}\) a hypotonic solution was obtained. In this condition, the interior of the RBC accumulates water that flows across the cell membrane into the cell, causing it to swell up to a quasi-spherical shape. Finally, these two RBC samples were deposited on Petri dishes and imaged by DH microscopy, after waiting about 10 min to guarantee sedimentation of the cells to the bottom of the Petri dish. The DH set-up, sketched in figure 2(a), is a Mach–Zehnder interferometer whose light source is a laser emitting 400 mW at 532 nm coupled in a fiber (not shown in the figure). The object arm is collimated with a diameter of about 2 mm and illuminates the RBCs in the Petri dishes. The light transmitted throughout the sample is then collected by a customized inverted microscope equipped with a water-immersion 60\( \times \) microscope objective, with numerical aperture of 1.20. The reference beam is recombined with the first one by a beam splitter, generating the digital hologram images of the samples that are recorded by a charge-coupled device camera (AVT Technologies) with 1024 × 1024 pixels of 6.7 \( \mu m \) pitches. We used a calibration object to calculate the effective magnification \( (M_\ell) \) of the recording system, namely \( M_\ell = 70.53 \), allowing \( p_x = 0.095 \, \mu m \) as the pixel size in the image plane. After recording of the the digital holograms, the QPIs were recovered using the classical MATLAB R2017b. The two QPIs used to verify the Talbot effect are reported in figure 3 for discocytes (figure 3(b)) and spherical-like RBCs (figure 3(c)).
3. Results and discussion

The self-imaging of RBC arrays is investigated by simulating the propagation of a plane wave through such arrays, thus searching for the intensity patterns at predicted Talbot lengths. As described in the previous section, QPI reconstructions of two RBC samples are considered to verify the existence of the Talbot effect for self-assembled cells, i.e. by waiting for all cells to settle to the bottom of the Petri dish. Of course, their positioning is almost random even if, at higher concentrations, RBCs create a monolayer of cells, as shown in figure 3(b) for discocytes and in figure 3(c) for spherical RBCs. It is important to note that RBCs try to arrange spontaneously in a hexagonal symmetry. Actually, the more similar the shapes and sizes of the RBCs the more precise the hexagonal arrangement is. In the case of discocyte RBCs, the total hexagonal symmetry seems to be more corrupted due to RBCs with smaller sizes or superposed ones. To investigate this aspect, we apply image segmentation using Otsu’s method in order to calculate binary masks of the RBCs within arrays, as shown in the green boxes in figures 3(b) and (c). The average period of RBC arrays can be calculated as the distance in pixel units between centroids of the central RBC and neighboring ones. Then we employ the pixel size in the image plane to convert these values to $\mu m$, giving $d_{disco} = 8.6 \pm 0.4 \mu m$ and $d_{sphero} = 9.4 \pm 0.6 \mu m$. Moreover, the form factor of these hexagons is usually lower than the nominal ones, i.e. $f < \sqrt{3} d_H$. This spread of positions is mainly caused by the non-homogeneous size and shape of the RBCs in the hexagonal array. Quantification of the deviation from perfect symmetry can be estimated by evaluating the average and standard deviation of surface areas (SAs) of RBCs, namely $SA_{disco} = 47.9 \pm 6.4 \mu m^2$ and $SA_{sphero} = 48.5 \pm 2.1 \mu m^2$. Also in this case, SAs are calculated from binary masks in figures 3(c) and (d) as the sum of the non-zero pixels within each RBC and then by converting pixel units to $\mu m^2$. By using the average periods calculated above, the primary Talbot length can be evaluated by equation (2), resulting in $Z_{T,disco} = 206.1 \mu m$ and $Z_{T,sphero} = 249.4 \mu m$. The complete analysis of the Talbot effect for discocytes and spherical RBCs is reported in figure 4. In particular, we simulate the light propagation through microlens arrays up to 5 mm from the array plane, supporting the search for the Talbot length with simulations and the TC metric. In this case, hexagonal arrays of simulated RBCs are designed to have cells of the same size and shape along with a similar symmetry to the original QPIs (see sub-figures within the red boxes in figures 4(b) and (d)). Thanks to the TC metric in figures 3(a) and (c), the focal planes can be easily detected (see sub-figures within green boxes in figures 4(b) and (d)). Moreover, the nominal self-imaging effect for experimental QPIs, at the calculated Talbot lengths, is reported in the sub-figures within violet boxes in figures 4(b) and (d). It is evident that such self-images provide noisy and smoothed focal spots of microlenses, as is also confirmed by looking the simulations, in which self-images appear with low contrast.

Actually, the TC trends show several peaks corresponding to intensity patterns with higher contrast. Among them, the intensity pattern having the highest spatial similarity compared with the focal plane image is chosen as the self-image. These intensity images are reported in figures 4(b) and (d) within the orange boxes, demonstrating that the self-imaging effect can be observed at distances equal to $Z_{disco} = 2886 \mu m$ and $Z_{sphero} = 2262 \mu m$. On the other hand, it is interesting to note that the self-imaging is obtained at distances very close to the integer multiples of the primary Talbot lengths, i.e. $Z_{disco} = 14.003Z_{T,disco}$ and $Z_{sphero} = 9.070Z_{T,sphero}$.
Figure 4. Proof of the Talbot effect for RBC arrays. In (a) and (c) the Tamura coefficient, calculated for each intensity reconstruction at distances in the range [0, 5] mm with a reconstruction step equal to 1 µm, is reported. In (b) and (d), the red boxes report QPIs for simulated (top) and experimental (bottom) RBC arrays for discocytes and spherocytes, respectively. Green boxes show the intensity reconstructions at the focal plane, while the violet boxes report the intensity images at the predicted Talbot lengths. Finally, orange boxes show the self-images with the highest image contrast.

Figure 5. Processing pipeline for investigating the Talbot effect for a simulated hexagonal arrangement with 100 real discocyte RBCs: (a) segmentation of RBCs from QPI reconstructions; (b) creation of a synthetic hexagonal arrangement with period \( d_H = 9.7 \) µm; (c) identification of the Talbot length by TC; (d) intensity reconstruction at the focal plane; (e) self-image at the Talbot length. In this case the intensity reconstruction at the Talbot length corresponds to the highest image contrast.

In several applications \([14–16]\), the Talbot effect is also used to investigate shape irregularities of some microlenses within the array but assuming perfect symmetry, i.e. a uniform array period. In general, the degradation of the self-images at the primary Talbot length is caused by (i) the imperfect symmetry of the microlens array and (ii) diversity of the microlens shapes. In our case, the results in figure 4 confirm the Talbot effect for self-assembled RBCs arrays, even if the poor quality of the self-imaging is mainly caused by variability of the array period. In fact, the simulation of discocytes and spherical RBCs in figure 4 demonstrates that, even with microlenses with ideal shapes and sizes, their arrangement needs to be as close as possible to the ideal hexagonal symmetry, otherwise a strong reduction of the image contrast of the intensity pattern at the Talbot length is observed. To overcome this limitation in investigating RBC samples, we performed a different analysis by synthesizing a perfect periodic hexagonal pattern. Each element of such
a synthetic array is made of experimental and segmented QPIs of diverse RBCs. Figure 5 reports the entire processing pipeline for investigating the Talbot effect for a simulated hexagonal arrangement with 100 real QPIs of discocytes. By using a fixed array period $d_y = 9.7 \, \mu m$, the Talbot length is $Z_T = 259.6 \, \mu m$. The TC trend shows the periodicity of the intensity patterns and the focal plane, and the corresponding self-images at the primary Talbot length are shown. This last result is reported as a phenomenological observation of the Talbot effect. Actually, the imaging characterization of RBC samples is based on the calculation of morphological features from images of each RBC separately.

The Talbot effect in RBC arrays permits the joint characterization of RBCs in a new way, thus creating a link between the diffraction theory underlying the Talbot effect and the study of biological samples. We demonstrate that all Talbot effects hold in the case of RBC arrays, thus validating the concept that a RBC can be considered as an optical biological lens as well as an optical element of a lenslet array. Moreover, new applications of blood analysis for diagnostic purposes, as in the case of identifying anemia [27], could be investigated by exploiting the optical features of the entire array and evaluating the sample in terms of self-imaging quality, as demonstrated in the experimental examples reported in figures 4 and 5. Even if this idea could be implemented in principle to perform specific diagnostic tests to detect irregularities in the shape and size (i.e. as biomarkers of blood diseases) of each element of the array, a quantitative analysis needs to be developed for specific diagnostic tests.

4. Conclusions

We demonstrate for the first time the Talbot effect in self-assembled and quasi-periodic arrays of RBCs under coherent light beam illumination. The proof is provided for RBCs having the classical biconcave shape and for spherical RBCs obtained by managing the osmolarity of the culture medium. We investigated the Talbot effect in the case of self-assembled RBCs in a monolayer and a perfect hexagonal array synthetically created with experimental QPIs of RBCs. In both cases the Talbot effect reveals the possibility of studying imperfect array periodicity as well as shape irregularities of RBCs. Almost all methods for characterizing RBCs work at the single-cell level, but this study investigates RBC samples by looking at all cells together, thus opening the way to characterize a biological material through its photonics properties.

ORCID iDs

Pasquale Memmolo @ https://orcid.org/0000-0002-9607-7728
Lisa Miccio @ https://orcid.org/0000-0001-9427-881X

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