Label-free tomography of living cellular nanoarchitecture using hyperspectral self-interference microscopy

Rongxin Fu,1,3 Ya Su,1,3 Ruliang Wang,1 Xue Lin,1 Kai Jiang,1 Xiangyu Jin,1 Han Yang,1 Li Ma,2 Xianbo Luo,2 Ying Lu,1 and Guoliang Huang1,2,*

1Department of Biomedical Engineering, the School of Medicine, Tsinghua University, Beijing 100084, China
2National Engineering Research Center for Beijing Biochip Technology, Beijing 102206, China
3Contributed equally as co-authors
*tshgl@mail.tsinghua.edu.cn

Abstract: Quantitative phase imaging (QPI) is the most ideal method for achieving long-term cellular tomography because it is label free and quantitative. However, for current QPI instruments, interference signals from different layers overlay with each other and impede nanoscale optical sectioning. Integrated incubators and improved configurations also require further investigation for QPI instruments. In this work, hyperspectral self-reflectance microscopy is proposed to achieve label-free tomography of living cellular nanoarchitecture. The optical description and tomography reconstruction algorithm were proposed so that the quantitative morphological structure of the entire living cell can be acquired with 89.2 nm axial resolution and 1.91 nm optical path difference sensitivity. A cell incubator was integrated to culture living cells for in situ measurement and expensive precise optical components were not needed. The proposed system can reveal native and dynamic cellular nanoscale structure, providing an alternative approach for long-term monitoring and quantitative analysis of living cells.

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1. Introduction

Long-term cellular tomography is an indispensable and powerful tool in both biomedical research and clinical diagnostics. Effective and continuous tomography of living cells, capable of deciphering native and dynamic cellular responses with nanoscale sensitivity, is critical to understanding long-term cellular morphology and metabolism [1]. To achieve effective and continuous cellular imaging, fluorescent microscopy beyond the diffraction limit has become a widely used option [2–4]. However, phototoxicity and photobleaching are inevitable obstacles using fluorescent imaging instruments. These two phenomena make fluorescent microscopy toxic to living cells and limit long-term imaging [5]. Moreover, the sample preparation process is often extremely complicated to import the fluorophore into the living cells. Similarly, challenges due to fluorophore labels also continue to hinder the wider implementation of confocal microscopy and two-photon microscopy [6]. To solve this problem, label-free imaging techniques provide an alternative solution. Phase contrast (PC) imaging and differential interference contrast (DIC) microscopy are two typical approaches. Unfortunately, both of these configurations can only provide qualitative images. Quantitative and label-free imaging requires further development.

Quantitative phase imaging (QPI) is a label-free imaging technique that focuses on precisely quantifying the phase shift caused by heterogeneous specimens [7]. This approach is able to reveal cellular nanoscale morphology without extraneous labels and, thus, has attracted a lot of attention [8]. However, although QPI has continued to improve, precise
nanoarchitecture imaging is still a challenging task. For QPI techniques, a phase shift results from summation of phase of electromagnetic waves as the incident light propagates through the entire imaging depth. Due to the spatial point spread function, interference signals of different layers are collected into the aperture of the objective lens and overlay with each other in the image plane. The compound phase shift cannot be resolved to reveal the original mappings of different layers [9]. For optically thick or multiple scattering specimens, the phase shift will even become imperceptible. Therefore, current QPI techniques cannot provide nanoscale axial resolution, though the interference phenomenon offers sub-nanometer sensitivity. Usually, the axial resolution of a QPI instrument is more than half of the wavelength, which impedes reconstruction results. Further, common QPI instruments use PC or DIC configurations to generate the coherent signal and exploit a spatial light modulator (SLM) or liquid crystal filter (LCF) to modulate the phase or wavelength. These components are all quite expensive, and precise adjustment is needed, which restrict the practical applications of QPI.

Interferometric reflectance imaging sensor (IRIS) is a label-free molecule detection platform that has gained extensive attention and interest [10–14]. This method records the interferometric reflectance spectrums for biomolecular mass measurements. Interference signals of different layers within the specimen can be separated into independent peaks in the Fourier domain [15]. Meanwhile, the phase information of each frequency reveals the tiny fluctuations in both the reflectance index and morphological structure. Based on this principle, various biosensors have been developed for nanoscale biomolecule detection, such as nucleic acid, protein, and biological nanoparticles detection [16–18]. In addition, HIS biosensors do not rely on precise instruments to generate solvable coherent signals, and thus, costly optical configurations or components are not necessary. Therefore, IRIS represents a promising method to solve the existing problems with QPI.

In this work, hyperspectral self-reflectance microscopy (HSM) is proposed to achieve label-free tomography of living cellular nanoarchitecture. A transparent specimen is placed on a monocrystalline silicon substrate. The incident light is reflected by both the heterogeneous cell and the silicon substrate. The reflected light generates a coherent signal that is a cosine function along the wavenumbers. A mathematical model describing the multi-layer interference signal was established, and a Fourier transformation-based algorithm is presented to reconstruct the nanoscale structure of the specimen. Moreover, based on this method, an integrated imaging system was developed with long-term cell culture ability. Compared to similar techniques, the proposed microscopy method possesses three major merits. First, the quantitative morphological structure of whole living cells can be acquired without any exogenous labels. Next, summation of phase is resolved, which contributes to nanoscale tomography of 89.2 nm intervals. Finally, a cell incubator was integrated to culture living cells for in situ measurement. The proposed microscopy method was able to reveal native and dynamic cell structure with nanoscale sensitivity, providing an alternative approach to long-term monitoring and quantitative analysis of living cells.

2. Materials and methods

2.1 HSM principle

In this paper, living cells (HeLa cells, bought from ATCC company, item number: EY-X0129) were cultured on a monocrystalline silicon substrate and the absorption was negligible. As Fig. 1(a) shows, the substrate could be divided into the silica layer (500 nm thickness) and the silicon layer. Living cells were immersed in the culture medium and grew on the silica substrate. The coherent signal was generated by three signals: the reflected light of the cell membrane $U_1$, the scattered signal of the cell content collected by the objective $U_2$, and the reflected light of the silicon wafer $U_3$. Thus, the intensity of the multi-layer interference signal equals [19]:
where $\lambda$ is the wavelength and $I(\lambda)$ is the coherent intensity of the corresponding wavelength. Assuming $U(\lambda)$ is the illumination field, according to Fresnel coefficient and optical phase theory, $U_i(\lambda)$ and $U_r(\lambda)$ equal [20]:

$$U_i(\lambda) = \frac{n_i-n_r}{n_i+n_r} U(\lambda)$$ (2) $$U_r(\lambda) = -2 \frac{n_0}{n_i+n_r} e^{-i \frac{\pi \lambda}{\lambda_0}} U(\lambda)$$ (3)

where $n_i$ and $n_r$ are the mean refractive index of the culture medium and the living cells. In this paper, we assumed that $n_i = 1.33$ and $n_r = 1.37$ [21]. $D$ is the thickness of the cell membrane.

$U_r(\lambda)$ is related to the refractive index distribution of the living cells. Assuming that the refractive index of a voxel $(x,y,z)$ is $n_r[1+n_i(x,y,z)]$ and the center wavelength is $\lambda_0$, the part of $U_r(\lambda)$ that propagates at solid angles within the numerical aperture of the objective equals [22]:

$$U_r(\lambda) = \frac{4n_i n_r}{i(n_i+n_r)} U(\lambda) \int -\frac{2\pi}{\lambda_0} n_i(x) \frac{i\Delta \lambda}{\lambda_0} dx$$ (4)

Here, the multi-layer summation of phase inside the living cells could be ignored as proven in previous publications [18]. Thus, the reflectance is:

$$R(\lambda) = \frac{(n_i-n_r)^2}{(n_i+n_r)^2} \frac{4n_i}{i(n_i+n_r)} e^{-\frac{i\Delta \lambda}{\lambda_0}} + \frac{8n_i n_r (n_i-n_r)}{i(n_i+n_r)^2} \int \frac{2\pi}{\lambda_0} n_i(x) e^{-\frac{i\Delta \lambda}{\lambda_0}} dx$$ (5)

It was obvious that $R(\lambda)$ is a constant plus a series of cosine functions. With a fast Fourier transform (FFT) algorithm, the values of $D$ and $n_i(x,z)$ could be resolved together.

![Image of a theoretical principle of HSM. (a) The mathematic description of the interferometric spectrum. (b) The schematic diagram of the whole setup. (c) The structure of the smart incubator.](image-url)
2.2 The integrated HSM system

In Fig. 1(b), the diagram of the integrated HSM system is shown. The basic optical setup is an epi-illumination microscope. All of the lenses were purchased from Edmund Optics (USA). The light source was a tungsten-halogen lamp (71PT250, Saifan, China), which provided a broadband spectrum of 200-1100 nm. Compared with common wide-field optical microscopy, the main difference is that the HSM system utilized a fiber-spectrograph (Maya2000, Ocean Optics, USA) system to collect the hyperspectral self-interference signal of the samples. The motion platform consisted of two parts: two pairs of step motors (E28H49-05, Haydon, USA) and lead screws (HGH25CA, Freud, China) for sample location and a two-dimensional piezo-ceramics platform (P-734, Physik Instrumente, Germany) for nanoscale scanning. A two-dimensional scanning task was performed so that the interferometric spectrum could be obtained pixel by pixel. In the following experiments, the scanning steps were set to 500 nm, and the integration time was set to 10 ms.

Long-term cell imaging without disturbance is very important for dynamic and long-term biomedical experiments. For example, dynamic quantitative imaging during cell proliferation and attachment is very important in biomedical research since this process is related to cancer growth and metastasis. Without the custom incubator, this long-term cell imaging experiments couldn’t be carried out because living cells couldn’t survive such a long time. In order to make the system more practical, we designed a custom incubator to culture the living cells. The smart incubator was integrated into HSM system, which is shown in Fig. 1(c). In this figure, we inclined the humidifier to make the heating film and the Pt100 sensor visible. The incubator is located on the motion platform and able to control temperature, humidity, and carbon dioxide density. A transparent cover made of indium-tin oxide-coated glass is not shown in the figure. The glass helped to heat the cover of the incubator to prevent condensation.

A control system was developed to control the motion platform and the smart incubator. Briefly, a Wheatstone bridge was used to transform the temperature to voltage signal. Then, the analog-digital converter (AD7705, Analog Devices, USA) contributed to transmitting the voltage to the microcomputer (STM32f103zet6, STMicroelectronics, Italy). A proportion-integration-differentiation algorithm was utilized to determine the output [23]. Sequence signals were offered to control the two motors with general purpose input/output configurations. A digital synthesizer (AD9854, Analog Devices, USA) helped to generate adjustable voltage and control the nanoscale platform.

2.3 Tomography reconstruction

First, standard wafer samples (500-nm thickness) were used for out-of-focus correction. We recorded the interferometric spectrums of the wafer while scanning the objective along the axial direction. Each spectrum had only one frequency component, thus displaying one peak after FFT. With this method, the relationship between the amplitude and focus plane position was detected. The amplitude correction factor for each out-of-focus plane was defined as the decay rate of the amplitude compared to that of the in-focus plane.

As shown in Eq. (5), the reflectance spectrums of the living cells were just a constant plus a series of cosine functions. Here, we utilized a bilinear interpolation function to reshape the reflectance spectrums to evenly distributed $1 \times 1024$ vectors. The FFT algorithm helped to calculate the frequency domain results. Then, each amplitude was divided by the corresponding correction factor. The largest peak in the frequency domain indicated the thickness of the cell membrane, and the amplitude of each frequency indicated the refractive index of each layer. Thus, information of each layer could be acquired, and tomography images of whole living cells could be reconstructed pixel by pixel.
3. Results

3.1 Principle verification with silica simulation samples

In this experiment, the resolution and sensitivity were determined, and silica phantom samples were exploited to verify the optical principle and test the reconstruction results of HSM. First, we simulated the reflectance spectra of different thicknesses and calculated the corresponding peaks after FFT processing. In Fig. 2(a), the theoretical peak positions of different thicknesses are shown. On average, when the sample thickness increased by 89.2 nm, the peak position increased by one. This indicated that the proposed method is able to distinguish two points with 89.2 nm intervals in height.

Next, we measured the reflectance spectra of wafers with different thicknesses and contrasted the results with the simulated spectra to determine the thickness of the silica layers. In Fig. 2(b), the measured results with the presented method and the theoretical values are shown in an error bar format. The theoretical values were measured by the method proposed in reference [24]. For 520.3 nm silica layer, the measurement was 519.7 ± 0.34 nm. For 681.3 nm silica layer, the measurement was 680.1 ± 0.33 nm. For 798.6 nm silica layer, the measurement was 799.4 ± 0.43 nm. For 910.8 nm silica layer, the measurement was 912.1 ± 0.22 nm. For 937.2 nm silica layer, the measurement was 937.6 ± 0.36 nm. The coefficient of determination was 0.9999. The maximal error was 1.3 nm and the maximal standard deviation was 0.43 nm.

Next, we designed and made silica phantom samples to verify the imaging ability of HSM. The phantom samples were squares with an edge length of 5 μm and the thickness of the squares was 500 nm. In Fig. 2(c-e), imaging results with three imaging methods are shown and in Fig. 2(f) the three-dimensional reconstruction result of the sample with HSM is demonstrated. The quantitative colors of HSM images indicated the thickness of the sample. In this figure, it was obvious that HSM presented the quantitative imaging results of transparent samples while DIC only offered the qualitative information.

(a)  (b)  (c)

(d)  (e)  (f)

Fig. 2. The axial resolution, sensitivity and reconstruction results of HSM. (a) The theoretical peak positions of different thicknesses. (b) The measurement results of wafers with different thicknesses. (c) Imaging results with scanning electron microscope. (d) Imaging results with DIC. (e) Imaging results with HSM. (f) The three-dimensional reconstruction result of the sample with HSM. The scale bar in (d) and (e) represent 5 μm.
3.2 Tomography imaging of a single living cell

In this experiment, tomography of a single living HeLa cell with HSM is shown and compared to conventional DIC imaging. In Fig. 3, imaging results from the two methods are shown. To better present the imaging results, we chose four typical tomography images, \( z = 3.3 \, \mu m \), \( z = 3.39 \, \mu m \), \( z = 4.73 \, \mu m \), and \( z = 4.82 \, \mu m \). The imaging results of different layers with HSM present more obvious differences due to the nanoscale axial resolution. The refractive index distributions of the different layers did not overlay with each other. The quantitative colors indicate the average refractive index, which ranged from 1.37 to 1.58, i.e., between the refractive index of water and protein [25].

![Fig. 3. Tomography of a single HeLa cell. (a) Imaging results of different layers with HSM. (b) Imaging results of different layers with DIC. The scale bars in the images represent 10 μm.](image)

3.3 Performance of the smart incubator

In this experiment, the performance of the proposed incubator was examined. In order to prove that the control quality of the smart incubator was satisfactory and the cell culture quality was reliable, a commercial incubator (C170, Binder, Germany) was used for comparison. As shown in Fig. 4, the temperature control, carbon dioxide concentration control, and culture performance of the two incubator were compared. For the temperature control of the proposed incubator, the rise-time was 1 min. The steady error was 0.42°C, and the overshoot was 0.8°C. For the temperature control of the commercial incubator, the rise-time was 40 min. The steady error was 0.5°C, and the overshoot was 0.5°C. The rise-time of the proposed incubator was much more rapid and the other values were similar to the commercial incubator. For the carbon dioxide concentration control of the proposed incubator, the rise-time was 3 min. The steady error was 0.262%. For the commercial incubator, the rise-time was 26 min. The steady error was 0.4%. Similarly, the rise-time of the proposed incubator was much more rapid and the steady errors were similar. Cell culture performance is also presented in Fig. 4. Both of the incubators could culture living cells for 24 h, and the cellular morphologies were nearly the same. We also took advantage of an automatic cell counter to contrast their performance. The live rates of the two incubators were both 100%. The cell concentration of the smart incubator was \( 9.38 \times 10^6 /mL \), while the cell concentration of the commercial incubator was \( 9.97 \times 10^6 /mL \). This means that the cell survivability of the custom incubator is comparable to that of a standard commercial one.
In this experiment, the main differences of the two incubators were the rise time of temperature control and carbon dioxide concentration control. Another obvious difference was that the temperature control curve of the smart incubator was rougher. These two phenomena were because the volume of the smart incubator was much smaller than the commercial incubator. The responses of the heating and carbon dioxide inlet were far more rapid for a smaller volume.

3.4 Dynamic imaging of HSM

In this experiment, dynamic tomography during cell division was demonstrated. We chose a HeLa cell that was dividing into two cells. Tomography images of four layers ($z = 3.3 \, \mu m$, $z = 3.39 \, \mu m$, and $z = 4.82 \, \mu m$) were recorded over 60 min. In Fig. 5, tomography of different layers during cell proliferation is shown. At 0 min, the cell had not divided into two isolated cells, the chromosomes existed, and the mean average refractive index of the chromosomes was more than 1.45 while the one of cytoplasm was only about 1.4. At 20 min, two cells were generated, and the chromosomes decondensed. The refractive index inside the cell decreased to ~1.4. At 40 min, the two cells completely separated. The refractive index nearby the membrane greatly increased, which indicated the cells synthesized protein and prepared for adherence [26]. At 60 min, the two cells had adhered to the substrate, and the contrast between the nucleus and the cell cytoplasm became distinct again. The average refractive index of the cell nucleus was 1.57, which approached that of protein, while the average refractive index of the cytoplasm was 1.39, which approached that of water.
4. Discussion

In Fig. 2(a), the axial resolution of HSM was demonstrated as the average thickness growth when the position of the peak after FFT increased one. In fact, the axial resolution could also be calculated theoretically with the frequency resolution definition of FFT:

\[
R_{\text{axial}} = \frac{f_c}{2n} = \frac{1}{2n \cdot 1.37 \times \left( \frac{1}{200} - \frac{1}{1100} \right)} = 89.21 \, \text{nm}
\]  (6)

Here, \( R_{\text{axial}} \) is the axial resolution, \( f_c \) is the frequency resolution of FFT and \( \tau \) is the sampling length. The maximal imaging thickness could be calculated with the maximal frequency of FFT:

\[
T_{\text{max}} = \frac{f_{\text{max}}}{2n} = \frac{f_{\text{max}}}{4n} = \frac{1024}{4 \times 1.37 \times \left( \frac{1}{200} - \frac{1}{1100} \right)} = 49.01 \, \text{nm}
\]  (7)

where \( T_{\text{max}} \) is the maximal imaging thickness, \( f_{\text{max}} \) is the maximal frequency of FFT and \( f_c \) is the sampling frequency.

It has to be pointed out that refractive index of biological is not homogenous, hence both the calculated axial resolution and the calculated maximal imaging thickness are merely approximate values. The precondition to confirm the two important performance indexes is to assume the refractive index of the living cell in 1.37.

In Fig. 2(b), the refractive index of the silica layer was assumed to be 1.47 [15]. The largest thickness measurement error of silica layer in this experiment was 1.3 nm. So the imaging sensitivity in optical path difference could be calculated as \( 1.3 \times 1.47 = 1.91 \, \text{nm} \). Here, we chose an indirect method to quantify the uncertainty in measured values of refractive index. In this experiment, we proved that the optical path difference sensitivity was 1.91 nm.
and the axial resolution was 89.2 nm. These two parameters meant that the proposed imaging method could tell the 1.91 nm optical path difference for every tomography and the thickness of each tomography was 89.2 nm. So the refractive index uncertainty could be calculated as $1.91 \text{ nm} / 89.2 \text{ nm} = 0.02$.

Due to the small volume of the proposed incubator, the temperature control and carbon dioxide concentration control were much more difficult. In Section 3.3, we demonstrated the detailed control performance to validate the incubator could culture living cells with the similar performance of the commercial incubator. The similar overshoot, steady error, cell concentration values proved this matter. The temperature rise time and carbon dioxide concentration rise time of the proposed incubator were much less. This was just due to the volume differences of the two incubators. But these two indexes are very important for the HSM system. Since the commercial incubators are always used without even a temporary break, the rise time is not critical. However, an imaging system is just used during the experiments, with frequent closures and restarts. No matter how long the experiment lasts, researchers have to power on the incubator and wait for the temperature and carbon dioxide concentration rise, then place the living cells. So in our system, researchers do not have to prepare for a long time. We believe that this will be very helpful in biomedical experiments.

In the Section 3.4, we chose the time interval of 20 min just because that after such an interval, the imaging results would change obviously during cell proliferation. In this experiment, the scanning area was $20 \, \mu\text{m} \times 100 \, \mu\text{m}$, the scanning steps were 500 nm and the exposure time for every spectrum data was 10 ms. So the total imaging time for the whole cell is 83.4 s, which is the minimum imaging time interval.

The maximum total imaging time is determined by how long the cells will cover the whole silicon substrate. The system can culture living cells and transport culture medium for the cells. But when the cells cover the whole substrate, they have to be digested with trypsin and transferred to a new substrate. This time is related to the cell type and initial cell concentration. Generally, the total imaging time can be longer than 24 hours. In this experiment, the broadband light source was powered on only when the spectrums was being collected, which only lasted for less than 1.5 min. For the other time, only white light was used for custom cell imaging with the CCD. So the light toxicity was negligible for long-term imaging. Figure 5 also proved that the cell proliferation and attachment could carried out, which validated that light toxicity didn’t affect the cell heavily.

5. Conclusion

Long-term cellular tomography of living cellular nanoarchitecture is an indispensable and powerful tool to decipher dynamic cellular morphology and metabolism. In this work, HSM is proposed to achieve label-free tomography of living cells. As a label-free method, HSM can be applied to reveal the nanoarchitecture of the whole cell with 89.2 nm axial resolution and 1.91 nm OPD sensitivity. The compound phase shift could also be resolved to reveal the original mappings of different layers, which makes this method an alternative choice for optically thick or multiple scattering specimens. HSM does not rely on expensive and precise optical components. A common epi-illumination setup could meet the imaging requirements. A cell incubator was integrated to culture living cells in an uninterrupted way, and thus, long-term and dynamic imaging can be achieved.

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