Light-sheet-based 2D light scattering cytometry for label-free characterization of senescent cells

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Abstract: A light-sheet-based 2D light scattering cytometer is developed for label-free characterization of senescent cells. The light-sheet provides an illumination beam with controlled thickness for single cell excitation, and 2D light scattering patterns are obtained by using a defocused imaging method. The principle of this cytometer is validated by distinguishing microspheres with submicron resolution. Automatic classification of senescent and normal cells is achieved at single cell level by using the support vector machine (SVM) algorithm, where a sensitivity of 89.1% and a specificity of 96.4% are obtained. Our results suggest that the light-sheet-based 2D light scattering label-free cytometry has the capability to perform size differentiation of beads with submicron resolution and to classify different groups of cells without fluorescent labeling, showing the potential for clinical diagnosis of senescence-related diseases.

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1. Introduction

Cell senescence plays an important role in understanding of age-related diseases and tumor-suppressor mechanism [1–3]. Conventional methods for the identification of senescent cells mainly depend on biomarkers [4, 5]. For example, the most extensively used method for senescent cell detection is to measure the SA-β-gal positive activity by histochemical staining [6, 7]. The presence of senescence-associated heterochromatin foci (SAHF) can also be used for the identification of senescent cells by detection of DNA dyes [8]. Cell senescence causes changes in cell morphology and cellular organelles, such as the nuclei, mitochondria and lysosomes [9]. Recently, the morphometric analysis depending on the changes in nuclear geometry and DNA distribution has been proposed as a new indicator of cell senescence, which can be measured in a laser scanning cytometry with the aid of fluorescence labeling [10].

A label-free method that can perform noninvasive identification of senescent cells is of great interest. Elastic light scattering has been demonstrated as an effective label-free method for cell or tissue analysis and disease diagnosis [11–13]. The light scattering intensity variation with the polar angle $\theta$, defined as one dimension (1D) light scattering, has been demonstrated to be sensitive to cell size and cellular contents [14, 15]. For example, Backman et al. have analyzed the size distribution and refractive index of cell nuclei by measurements of the backward scattered light [16]. However, the biological cells are complex due to the various cellular organelles and more information is needed to better characterize the cells. Two-dimension (2D) light scattering (related to both the light scattering polar angle $\theta$ and azimuthal angle $\phi$) may provide more details about the cellular and subcellular structures for label-free cell characterization. In 2007, Su et al. have demonstrated for the first time that 2D light scattering patterns contain mitochondrial information of cells, showing the potential of 2D light scattering technique for cellular analysis [17]. In terms of flow cytometry, 2D light scattering technique has recently been integrated with the microfluidic cytometry for label-free analysis of single cells in a fluidic stream [18, 19].

Unlike the label-free cytometry by measurements of autofluorescence [20, 21], the obtaining of 2D light scattering patterns in cytometry requires high signal-to-noise ratio (SNR) due to the property of elastic light scattering. Usually, the background noise in a light scattering system is caused by the refractive index mismatch at the sample-medium interface, or light scattering from other scatterers. These undesirable signals may deteriorate the image quality and lead to low efficiency of parameter extraction from the 2D patterns. In order to improve the SNR of 2D light scattering flow cytometry, an aperture based method has been proposed for the obtaining of weakly scattered light from cells, where the aperture could be the numerical aperture of an objective [18, 22] or an aperture of microfabricated window [18]. The aperture based method has been further developed into microscope-based microfluidic cytometry by our group [19].

Another issue in novel flow cytometer development concerns the light illumination of cells in flow. In conventional flow cytometry, the incident laser light is manipulated into an ellipsoidal illumination structure by using complex optics, and single cells in a sheath flow are illuminated by end-fire coupling [23]. Compared with the laser spot illumination with an ellipsoidal structure in conventional flow cytometer, a focused laser beam has also been used for the illumination of cells in flow with a spherical structure [24]. Prism coupling or fiber coupling technique has been adopted in flow cytometry developments [18, 25]. These methods however have no effective control on the shape of the illumination beam as in conventional cytometry. Recently, light-sheet illumination has been developed to replace the point or full-field illumination in fluorescent microscopy, and performs optical sectioning of cells or tissues by exciting only the fluorescent molecules in the focal plane. Since Huisken and his colleagues applied the light-sheet technique for reconstruction and visualization of developmental embryo [26], light-sheet fluorescent microscopy has been shown with wide...
applications in biomedicine such as the long-time imaging in developmental biology [27], and the real-time tracking of molecule or cell dynamics in living tissues [28, 29]. In flow cytometry, Chan et al. have shown the sectioning of fluorescent ocean phytoplankton by adopting the light-sheet illumination [30]. The light-sheet method may be applied to 2D light scattering cytometry to enhance the SNR as a controllable illumination for label-free cell analysis.

In this manuscript, we develop a light-sheet-based 2D light scattering cytometric technique for label-free characterization of senescent cells. The cytometer presented here adopts a light-sheet illumination to restrict the excitation volume, and thus the background noise can be reduced. Another advantage of this cytometer is that the light sheet thickness is readily controllable for diverse samples with different sizes. The particles or cells are observed in suspension in the light-sheet-based label-free cytometer, and the focused image as well as the defocused light scattering pattern of a scatterer are obtained via the same microscope objective with a numerical aperture (NA) of 0.4. In this work, 2D light scattering patterns from different polystyrene beads are obtained to demonstrate the capability of our light-sheet-based cytometry for size differentiation with submicron resolution. The experimental results from standard microspheres agree well with simulations based on Mie theory. Two-dimensional light scattering patterns of normal human fibroblasts (NHF) and senescent human fibroblasts (SHF) are obtained by the verified light-sheet-based label-free cytometer. Two parameters are extracted from the obtained 2D patterns, which are then analyzed by support vector machine (SVM), a supervised machine learning algorithm [31]. The high accuracy rate achieved for the classification of NHFs and SHFs demonstrates that our 2D light scattering cytometry with light-sheet illumination may help for the understanding of age-related diseases and antitumor mechanism by label-free observation of senescent cells.

2. Experiments and method

2.1 Experimental setup

A schematic of the light-sheet-based 2D light scattering static cytometer is illustrated in Fig. 1. The incident beam with a wavelength of 532 nm and a diameter of 1.05 mm is generated by a diode pumped solid state (DPSS) laser (Frankfurt, Germany). A thin light sheet is obtained by using a cylindrical lens to excite the sample in suspension on a sandwiched chip, which is fixed on a three-axis translation stage. The chip is made with two 170 μm (thickness) glass sheets that serve as the bottom and top layers and two coverslips as gaskets, forming a waveguide. The dimension of the waveguide is 24mm×10mm×0.17mm (length, width and height, respectively), as shown in the inset of Fig. 1. Two-dimensional light scattering patterns are obtained by a CMOS sensor (Canon, Japan) via an objective lens with a numerical aperture (NA) of 0.4 (Olympus, Japan). If not otherwise specified, the integration time used for the obtaining of the 2D patterns is 0.04 s. The 2D pattern detection system is integrated onto a reconfigured inverted microscope (Olympus, Japan). Experimental 2D light scattering patterns can be further analyzed for automatic cell classification by machine learning algorithms.
Fig. 1. Schematic of the light-sheet-based 2D light scattering static cytometer. A collimated laser beam is reshaped by a cylindrical lens to form a light sheet. The sample in a waveguide as shown in the inset is illuminated by the thin light sheet. A CMOS sensor is used to obtain 2D light scattering patterns via a microscope objective with an NA of 0.4. The experimental patterns are sent to computer and are analyzed for particle sizing and cell differentiation.

The propagation of the scattered light from a single scatterer is illustrated in Fig. 2. The single scatterer in solution is excited by a light sheet with thickness \( d \) (measured at the beam waist). The thickness of the light sheet is assumed to be uniform in the range that is two times as long as the Rayleigh length. There are various methods to form desirable thickness of the light sheet [32–34]. Here we use cylindrical lenses with different focal lengths to obtain expected light sheet thickness according to the size of a microsphere or cell. In this work, the thickness of the light sheet is designed to be at least twice the size of the scatterer. If the thickness of the light sheet is narrower compared with the size of the scatterer, it would not be able to excite the whole scatterer.

Fig. 2. Illustration of the scattered light ray path from a single scatterer excited by a light sheet. The light sheet is with a thickness of \( d \) at the beam waist. The 2D light scattering pattern is detected by an optical objective working in positive defocusing mode. The angle \( \theta \) denotes the scatter polar angle and \( \alpha \) is the maximal half-angle of the cone of light that can enter the microscope objective. The refractive index of solution, glass and air are indicated as \( n_1 \), \( n_2 \) and \( n_3 \), respectively. The top and bottom glass layers are both 170 \( \mu \)m in thickness. (Not to scale)

As shown in Fig. 2, the side scattered light passes through the solution (\( n_1 = 1.334 \)), a layer of glass (\( n_2 = 1.51 \)) and air (\( n_3 = 1.00 \)) onto the microscope objective. In this case, the
scatter angle $\theta$ is obtained from approximately 72.5° to 107.5° considering the optical layout of our cytometer. The microscope objective can work in focusing mode and defocusing mode for the obtaining of images and 2D patterns of the scatterers, respectively [25]. The 2D light scattering patterns are obtained in the positive defocusing mode with $\Delta z = 200 \mu m$ in this work.

The light-sheet illumination is a decent excitation approach that can benefit our 2D light scattering cytometry. Specifically, the light-sheet beam effectively narrows the excitation volume and only the single scatterers that are of interest will be excited. Thus the background noise is decreased by eliminating light scattering from other scatterers and the refractive index mismatch especially at the sample medium-glass interface. In this work, we are using a microscope objective with an NA of 0.4 for the collection of the 2D patterns, which has a limited field of view (approximately 500 µm in radius). Considering that the scatterers on chip are located in an area of about 10 mm by 10 mm, the light scattering that occurs at the edge of the chip due to the incidence of the light sheet from air to the sample medium could be eliminated. Also, the thickness of the light-sheet beam can be controlled for different scatterer sizes. The adopting of light-sheet illumination in label-free cytometry not only enhances the SNR for 2D light scattering measurements, but also provides a method for reshaping the beam for effective illumination in a liquid solution.

2.2. Sample preparation

The Rhodamine 6G fluorescent dye (Life Technologies, USA) with an excitation wavelength of 535 nm and an emission wavelength of 575 nm was diluted to a density of 2.61 µM in ultrapure water. The standard polystyrene microspheres (Bangs Laboratories, USA) with diameters of 3.87 µm and 4.19 µm were diluted with ultrapure water and sonicated for 5 minutes.

Normal human fibroblasts were prepared as described [35, 36]. The cells were obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing). They were maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin-streptomycin and 4 µg/mL basic Fibroblast Growth Factor (bFGF) in a humidified 5% CO$_2$/95% air atmosphere at 37°C. For the obtaining of SHFs, the NHFs (the 17th passage) were treated with 600 µM H$_2$O$_2$ for 7 days. Both NHFs and SHFs were fixed with Immunology Staining Fix Solution (Beyotime, China) for 15 mins at room temperature. The cells were then washed three times with phosphate buffer saline (PBS), and were resuspended in PBS before the light scattering experiments.

3. Results and discussion

3.1 Measurements of the light sheets

To measure the thickness of the light sheet achieved in the experiments, the Rhodamine 6G solution was used for the visualization of the light sheet profile. An optical bandpass filter (Olympus, Japan) was employed to allow only the emission wavelength to be detected. Cylindrical lenses with focal lengths of 25.4 mm and 150 mm were used to generate the designed thicknesses, respectively. The corresponding effective NA is 0.02 for 25.4 mm lens, and is 0.0035 for the 150 mm lens. The beam profiles generated with these two lenses are shown in Figs. 3(a) and 3(b), respectively. The diameter of the light-sheet beam waist along the dashed line in Fig. 3(a) is about 13.76 µm, which was measured at full width at half maximum (FWHM) as plotted in Fig. 3(c); and that of Fig. 3(b) is 53.32 µm as shown in Fig. 3(d). The Rayleigh length is about 475 µm for the light sheet with a thickness of 13.76 µm and is up to 10 mm for the 53.32 µm light sheet. As shown in Figs. 3(c) and 3(d), the intensity of the light sheet drops to zero at the medium-glass interface (the thickness of the solution layer is about 170 µm) for both of the two light sheets. In this case the light scattering that
could occur at the medium-glass interface will be greatly reduced as compared with the illumination with an inserted optical fiber where the light will diverge quickly in the sample medium [25]. The light sheet has a large extent in the x and y directions, which may cause background scattering that can be detected by the microscope objective with an NA of 0.4 (although the field of view of the microscope objective is with a radius of about 500 μm). To further reduce the background scattering due to the large extent of the light sheet in the x and y directions, a slit could be incorporated into our system for more sensitive detections.

Fig. 3. Measurements of the light sheet thickness in the 2D light scattering label-free cytometry. (a) shows the image of a light sheet in the Rhodamine 6G solution generated by a cylindrical lens with a focal length of 25.4 mm, and (b) is from a cylindrical lens with a focal length of 150 mm. Scale bar: 50 μm. (c) and (d) show the measured thickness of the light sheets in (a) and (b), respectively.

In this work, the light sheet with a thickness of 13.76 μm or 53.32 μm is used for the illumination of microspheres and human fibroblasts, respectively. The selection of a light sheet with proper thickness can guarantee that the scatterer is fully excited with a reduced background noise along the z axis of the light sheet.

3.2 Microsphere size differentiation by light-sheet-based 2D light scattering cytometry

The operation of our newly developed light-sheet-based 2D light scattering cytometry was verified by performing experiments on single polystyrene microspheres with diameters of 3.87 μm and 4.19 μm. Figures 4(a) and 4(b) are the microscope images of 3.87 μm and 4.19 μm microspheres obtained via a 20× microscope objective with an NA of 0.4. The optical resolution of this microscope objective is approximately 810 nm at 532 nm excitation, making it challenging to distinguish these microspheres by conventional microscope imaging. Figures 4(c) and 4(d) show the experimental 2D light scattering patterns of Figs. 4(a) and 4(b), respectively. It is obvious that the scattering patterns are with different distributions of fringe structures. The pattern of 3.87 μm microsphere in Fig. 4(c) presents 7 bright fringes, while for 4.19 μm microsphere there appears 8 bright fringes in Fig. 4(d). Simulated 2D light scattering patterns of 3.87 μm and 4.19 μm microspheres were obtained by using our Mie theory based algorithm, as shown in Figs. 4(e) and 4(f), respectively. Note that both the polar angle and the azimuthal angle are from 72.5° to 107.5° for Figs. 4(e) and 4(f), as the same as the experimental 2D patterns. For the simulations, the refractive index of the microsphere is 1.591 at the excitation wavelength of 532 nm, and the refractive index of surrounding medium is assumed to be 1.334. The simulated 2D patterns agree well with the experimental results in terms of pattern distributions.

A Fourier method was adopted for further analysis of the 2D patterns quantitatively [18]. In this work, Fourier transforms were performed on the cross section scanning results of the 2D patterns in Figs. 4(c)-4(f), that is, in the polar angle range of 72.5° to 107.5° at an azimuthal angle of 90°. Figure 4(g) shows the Fourier spectra of 3.87 μm and 4.19 μm microspheres. For the 3.87 μm microsphere, the typical frequency of the Fourier spectra is
The typical frequency of the experimental pattern is the same with the simulated result, both at 1.4342 (1/degree) for 4.19 μm microsphere. Since the step size of the Fourier spectra is 0.0138 (1/degree), the experimental results agree well with the simulated ones within error. The typical frequencies of the experimental results of the 3.87 μm and 4.19 μm standard microspheres have a difference of 0.1379, which is 10 times that of the Fourier spectra step size. Noting that the two kinds of microspheres we used here have a size difference of 320 nm, and our light-sheet based 2D light scattering cytometry demonstrates the capability for size differentiation with submicron resolution by Fourier analysis.

3.3 Classification of senescent cells by light-sheet-based 2D light scattering cytometry

The percentages of senescent cells to the total number of cells of H2O2 treated human fibroblasts and natural human fibroblasts (control sample) were evaluated by testing the
senescence-associated acidic β-galactosidase (SA-β-gal) activity before the light scattering experiments. The SA-β-gal activity was evaluated using the SA-β-gal staining kit (Cell Signaling Technology, USA) following the manufacturer’s instructions. The results are shown in Figs. 5(a) and 5(b). The percentage of senescent cells is about 95% for the H_2O_2 treated sample, while it is below 1% for the control sample, as shown in Fig. 5(c). Thus we assume the H_2O_2 treated human fibroblasts as senescent cells, and the control sample as presenescent cells.

![Image](image1.png)

Fig. 5. Evaluation of the human fibroblasts senescence rate for the control sample and H_2O_2 treated sample. (a) is the representative image of the control group and (b) is for the H_2O_2 treated sample. Scale bar: 100 μm. (c) shows the percentages of SA-β-gal positive cells of the two samples.

Using our light-sheet-based label-free cytometry, we acquired 2D light scattering patterns from both the NHFs and the SHFs, each for 55 patterns. Figures 6(a) and 6(b) show the representative phase contrast microscope images of an NHF and an SHF. The corresponding 2D light scattering patterns are presented in Figs. 6(c) and 6(d). It is noticed that the patterns of both NHF and SHF are dominated by speckles. Analyzing of these speckles may be used for label-free classification of NHFs and SHFs.
Fig. 6. Representative 2D light scattering patterns of the NHF and SHF obtained with the light-sheet-based label-free cytometry. (a) and (b) are the microscope images of an NHF and an SHF. Scale bar: 20 μm. (c) and (d) show the corresponding 2D light scattering patterns.

The average area of the speckles in a 2D pattern varying with its speckle number is plotted in Fig. 7, where the open circle and square signs denote the results of NHFs and SHFs, respectively. The solid circle and square signs show the results of NHF (Fig. 6(a)) and SHF (Fig. 6(b)), respectively. From Fig. 7, the NHFs distribute within the upper left area, while in the case of SHFs the distribution presents a relatively wider range located at the lower right area. The mean and standard deviation (SD) of both the average areas and the speckle numbers for the groups of NHFs and SHFs were calculated. The results are shown in Fig. 7 as denoted by solid and open triangle signs, respectively. It is noticed that the group of NHFs have 177 ± 28 speckles with the average area of 211 ± 51 pixel², while for the group of SHFs the speckle number is 275 ± 51 and the value of average area is 147 ± 31 pixel². These results show that the groups of NHFs and SHFs distributed within different ranges in terms of the two parameters (average area of the speckles and speckle number in a 2D pattern), and can be distinguished from each other.
Fig. 7. Differentiation of NHFs and SHFs by light-sheet-based 2D light scattering label-free cytometry. The average area of the speckles in a 2D pattern varying with its speckle number is plotted, as shown in open circle and square signs for NHFs and SHFs, respectively. The solid circle and square signs denote the results of NHF in Fig. 6(a) and SHF in Fig. 6(b), respectively. The mean and SD for the average areas and speckle numbers of the groups of NHFs and SHFs show that these two groups of cells can be differentiated.

In order to perform single cell characterization automatically, SVM was adopted to analyze the data of NHFs and SHFs. The SVM algorithm is particularly effective in the case of small training data sets, and is able to construct a hyperplane to optimally separate different classes of data points [31, 37, 38]. In our work, the SVM classifier is based on a linear kernel function with 5-fold cross validation. The total 110 training samples from the NHFs and SHFs are grouped randomly into 5 subsets with 22 samples each, where the SHFs are set as positive group and the NHFs as negative group. For each supervised learning process, four subsets are used as training data, and the fifth subset is tested automatically. The results for the characterization of NHFs and SHFs at single cell level are shown in Table 1. In Table 1, the sensitivity is defined as the percentage of SHFs that are correctly identified, and the value is 89.1%. The specificity that describes the recognition accuracy of NHFs is obtained as 96.4%. By adopting the automatic classification of NHFs and SHFs via SVM machine learning algorithm, an overall classification accuracy of 92.7% is achieved. The parameter of AUC, short for area under curve, is commonly used to evaluate the performance of a classifier. The good performance of the SVM for the automatic classification of NHFs and SHFs is solidified by a high value of AUC as of 0.967. This demonstrates that our light-sheet-based 2D light scattering cytometer can perform label-free single cell characterization of SHFs with good accuracy rates.

| Type | Total Number | Correct Number | Specificity | Sensitivity | Accuracy | AUC  |
|------|--------------|----------------|-------------|-------------|----------|------|
| NHFs | 55           | 53             | 96.4%       | 89.1%       | 92.7%    | 0.967|
| SHFs | 55           | 49             |             |             |          |      |

4. Conclusion

A light-sheet-based 2D light scattering label-free cytometer was developed for the analysis of single cells or particles. Compared with conventional cytometry, the light-sheet-based cytometry has an excitation source with a controlled thickness and a relatively long Rayleigh range. This enhances the SNR for the measurements of single cells, especially for the label-
free 2D light scattering acquisitions. Experiments on standard polystyrene microspheres of 3.87 and 4.19 µm in diameter were performed, and 2D light scattering patterns were obtained by using a 20× microscope objective with an NA of 0.4, which has a resolution of about 810 nm for size differentiation in conventional microscopy. The 2D light scattering patterns of these two kinds of microspheres are distinctive, and Fourier analysis showed that size differentiation with submicron resolution of less than 320 nm can be achieved by the light-sheet-based 2D light scattering cytometer.

A method for label-free identification of senescent cells may help our understanding of age-related diseases and antitumor mechanism. The light-sheet-based 2D light scattering label-free cytometer was applied to characterize the NHFs and SHFs, which were tested by conventional histochemical staining. Two parameters were extracted from the 2D patterns, namely the average area of the speckles in a 2D pattern and its speckle number, which can be used for the differentiation of the NHFs and SHFs into two groups. In order to perform single cell identification automatically by our light-sheet-based 2D light scattering label-free cytometry, machine learning method has been adopted to analyze the 2D patterns. A sensitivity of 89.1% for the identification of SHFs and a specificity of 96.7% for the identification of NHFs were achieved by using SVM. Our light-sheet-based 2D light scattering label-free cytometry has promise for label-free characterization of senescent cells for disease diagnosis.

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