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Multimodal Spectral Imaging of Cells Using a Transmission Diffraction Grating on a Light Microscope

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Abstract

A multimodal methodology for spectral imaging of cells is presented. The spectral imaging setup uses a transmission diffraction grating on a light microscope to concurrently record spectral images of cells and cellular organelles by fluorescence, darkfield, brightfield, and differential interference contrast (DIC) spectral microscopy. Initially, the setup was applied for fluorescence spectral imaging of yeast and mammalian cells labeled with multiple fluorophores. Fluorescence signals originating from fluorescently labeled biomolecules in cells were collected through triple or single filter cubes, separated by the grating, and imaged using a charge-coupled device (CCD) camera. Cellular components such as nuclei, cytoskeleton, and mitochondria were spatially separated by the fluorescence spectra of the fluorophores present in them, providing detailed multi-colored spectral images of cells. Additionally, the grating-based spectral microscope enabled measurement of scattering and absorption spectra of unlabeled cells and stained tissue sections using darkfield and brightfield or DIC spectral microscopy, respectively. The presented spectral imaging methodology provides a readily affordable approach for multimodal spectral characterization of biological cells and other specimens.

Index Headings

Spectral imaging; Single cell; Transmission diffraction grating; Light microscopy; Spectroscopy; Fluorescence; Brightfield; Darkfield; Differential interference contrast; DIC

Introduction

Light microscopy and spectroscopy are used in various modalities for the analysis of biomolecular composition and structure of biological cells. For example, fluorescence microscopy is used for localization of fluorescently labeled or natively fluorescent biomolecules in cells,¹ while fluorescence spectroscopy yields information on the spectral properties and amounts of analyzed molecules.² Other forms of microscopy (e.g., brightfield, darkfield, phase contrast, and differential interference contrast (DIC) microscopy)³ and spectroscopy (e.g., ultraviolet–visible (UV–Vis) spectroscopy)⁴ are also used for the analysis of biomolecules in cells. Because microscopy and spectroscopy provide complementary data, it is very useful to combine these two methodologies and

simultaneously monitor changes in localization of biomolecules inside biological cells on the basis of their spectral properties.

Spectral imaging, a methodology that couples spectroscopy and microscopy, is a hyphenated technique that has received much scientific attention over the past ten to fifteen years.^{5,6} The technique is based on microscopes that employ customized optical filters, spectral dispersion elements (e.g., a diffraction grating or an interferometer),^{5,6} or tunable filters (e.g., an acousto-optic or a liquid-crystal tunable filter)^{7,8} to record images as well as spectra of molecules in individual cells and other specimens. Due to the development of a plethora of fluorescent probes, fluorescence spectral imaging became very useful for multi-color analysis of cells and cellular organelles. Compared to conventional microscopes that use various optical filter sets to specifically select fluorophores in cells, fluorescence spectral imaging offers the advantage of simultaneous multi-colored imaging of cells. Applications of fluorescence spectral imaging include analysis of biomolecules in cells and cellular organelles labeled with multiple fluorophores,^{8,9} monitoring of fluorescent molecules whose spectra temporally change due to environment or fluorescence energy transfer (FRET),^{6,10} labeling of DNA in chromosomes with multicolored fluorescent dyes for detection of chromosomal aberrations by spectral karyotyping (SKY),^{5,11,12} and spectral identification of single molecules and fluorescent nanospheres.^{13–15} Other forms of spectral imaging that can measure scattering and absorption spectra from biomolecules, nanoparticles in cells, and dye specimens are also gaining popularity.^{16–18} Hence, spectral imaging finds important applications in biochemical, biophysical, and biomedical research.

Currently, there are a few spectral microscopes commercially available to scientists for measurement of spectra of cells, cellular organelles, and other specimens. For example, a spectral microscope that uses a custom fluorescence filter cube and an interferometer for fluorescence spectral imaging is used for karyotyping of human and mouse chromosomes.¹² Although spectral imaging instruments are essential for the measurement of spectra of fluorophores in analyzed cells or colorimetric investigation of tissue specimens, they are not readily affordable for many laboratories. Therefore, several recent studies have described alternative but efficient ways to record spectral information from microscopic specimens using a diffraction grating. A high-throughput spectral imaging method based on a transmission diffraction grating was used to measure fluorescence spectra of single molecules and fluorescently labeled cells that flow through a capillary.^{9,13,14} Another setup based on a reflective diffraction grating was developed recently to record absorption spectra of dyes and was featured as a simple and inexpensive alternative to commercially available spectral microscopes.¹⁸ The presented work outlines a spectral imaging setup based on a transmission diffraction grating that can be used for fluorescence as well as absorption (colorimetric) and scattering (darkfield) spectral imaging of cells.

While transmission gratings have been used in microscopy and spectroscopy for a long time, their applications for single-molecule and single-cell fluorescence imaging are recent.^{9,13–15} However, high-resolution and high-magnification multimodal spectral imaging of cells through a transmission diffraction grating has not yet been investigated. Because most standard light microscopes do not have spectral imaging capabilities, it is challenging to perform imaging of biological cells and other specimens when high-resolution spectral imaging studies are needed. With a significant increase in the applications of multi-color fluorescence, darkfield, and DIC spectral imaging, it is important to investigate how a microscope containing an inexpensive transmission diffraction grating can facilitate detailed multimodal spectral analysis of biomolecules in individual cells. Moreover, the spectral imaging methodology based on the transmission diffraction grating could complement or improve the data obtained by other spectral imaging techniques.

Therefore, we report the development of a multimodal spectral imaging methodology based on a transmission diffraction grating and its application for spectral analysis of biological cells. The methodology is developed for several microscopy modes as well as combinations thereof. Fluorescence spectral imaging of yeast, bovine, and mouse cells, darkfield spectral imaging of human cells, and brightfield and DIC spectral imaging of stained tissue sections, dyed nanoparticles, and free dyes were performed using the presented methodology.

Experimental

Chemicals and Materials

Microscope glass slides, coverslips, hematoxylin, cell growth media, and ChromoSphere™ red dyed microspheres ($d = 50 \mu\text{m}$) were purchased from Thermo Fisher Scientific (Pittsburg, PA). Fluorescent dyes 4',6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), rhodamine 123, and rhodamine isothiocyanate (RITC) were purchased from Sigma (St. Louis, MO). Yeast from *Saccharomyces cerevisiae*, type II, was also obtained from Sigma. Polystyrene nanoparticles with diameter of 800 nm were from Bangs Laboratories. FluoCells® prepared slide #1 containing bovine pulmonary artery endothelial (BPAE) cells and FocalCheck™ fluorescence microscope test slide #3 were from Invitrogen Molecular Probes (Eugene, OR). In the BPAE cells, nuclei were labeled with DAPI, actin filaments in the cytoskeleton were labeled with Alexa Fluor 488-phalloidin, and mitochondria were labeled with MitoTracker® Red CMXRos. FocalCheck™ fluorescence microscope test slide contains blue, green, orange, red, and deep red fluorescent particles with diameters of $2.5 \mu\text{m}$. Human cheek epithelial cells were noninvasively sampled onto a glass slide from a healthy volunteer after receiving consent. Hematoxylin and eosine (H&E) stained slides were purchased from Carolina Biological Supply (Burlington, NC).

Yeast Cell Labeling

Yeast cells were washed three times with $1\times$ phosphate buffered saline (PBS) solution. After washing, cells were suspended in PBS and fluorescent labels were added to the cells in the following concentrations: $1 \mu\text{L}$ of $1 \mu\text{g/mL}$ DAPI solution in PBS, $5 \mu\text{L}$ of a 1 mg/mL FITC solution in DMSO, and $5 \mu\text{L}$ of a 1 mg/mL RITC solution in DMSO. Cells were incubated with the dyes in the dark for 30 minutes. After the incubation, cells were washed with PBS three times again and resuspended in 1 mL of PBS solution.

Mouse Cell Growth and Labeling

Mouse vascular endothelial cells were cultured on $22 \text{ mm} \times 22 \text{ mm}$ coverslips in six-well plates filled with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin. The cells were grown in the incubator under 5% CO_2 at 39°C . For labeling, 2 mL of media was mixed with $20 \mu\text{L}$ of $10 \mu\text{g/mL}$ DAPI solution in water, $10 \mu\text{L}$ of a 1 mg/mL rhodamine 123 solution in water, and $10 \mu\text{L}$ of a 0.6 mg/mL RITC solution in water. Dye mixture was added onto the coverslip containing cells, which was rinsed with PBS, and the cells were incubated with the dyes at 37°C for 90 minutes. Then, the dye solution was discarded and the coverslip was washed twice with PBS and culture medium for 10 minutes.

Preparation of Microscope Slides

Slides containing yeast cells were prepared after $1 \mu\text{L}$ of yeast cell suspension in PBS was delivered on a microscope slide and covered with a coverslip. Upon sampling, human cheek epithelial cells were spread on the glass slide and covered with a coverslip. Mouse vascular endothelial cells grown on a coverslip were washed twice with PBS, and the coverslip was attached to the microscope slide containing a small drop of PBS and antifade solution using

double-sided tape. For prolonged storage of slides, the coverslips were sealed around the edges with nail polish. Particle suspensions were dispersed in water and sandwiched between a microscope slide and a coverslip. Other slides were used as received from the manufactures.

Spectral Imaging

Cells were imaged using a Nikon Eclipse 80i upright microscope equipped for simultaneous work in the epi-fluorescence and transmission light microscopy modes (i.e., DIC, darkfield, or brightfield mode). 10× Plan Fluor (numerical aperture, NA = 0.3), 40× Plan Fluor (NA = 0.75), and 100× Oil Plan Apo (NA = 1.4) objectives were used for imaging by fluorescence, DIC, and brightfield microscopy. A 40× Plan Fluor and a 100× Oil Plan Fluor objective with an iris diaphragm (NA = 0.5–1.3) were used for darkfield microscopy. For fluorescence imaging, the following filter cubes were used: DAPI (excitation filter: 360BP40; emission filter 460BP50), FITC (480 BP40; 535BP50), TRITC (545BP30; 620BP60), Texas Red (560BP55; 645BP75), and triple DAPI/FITC/Texas Red (excitation: 395–410 nm, 490–505 nm, and 560–585 nm; emission: 450–475 nm, 515–545 nm, and 600–652 nm). One high-resolution oil condenser (NA = 1.4) was used for cell imaging by DIC and brightfield microscopy, while another oil condenser (NA = 1.20–1.43) was used for darkfield microscopy. Optics for DIC microscopy included appropriate DIC prisms, a polarizer, and an analyzer. An X-Cite 120 illuminator (EXFO Life Sciences, Mississauga, Canada) coupled to a liquid light guide was used for illumination of cells for fluorescence microscopy, while a halogen lamp was used for illumination of cells in DIC, brightfield, and darkfield microscopy. A 45 mm green bandpass filter (546BP20) was used for selective illumination for darkfield microscopy and occasionally for DIC microscopy. Images of cells were acquired using a Photometrics CoolSNAP ES² charge-coupled device (CCD) camera (Tucson, AZ). The camera contained an imaging array consisting of 1392 × 1040 pixels, and the size of each pixel is 6.45 μm × 6.45 μm.

The transmission grating containing 70 grooves/mm was purchased from Edmund Scientific (Barrington, NJ). The spectral imaging methodology used is similar to the one used previously for fluorescence imaging of single molecules.¹⁴ Briefly, the only modification to a conventional upright light microscope was incorporation of a diffraction grating in the light path. Initially, a transmission grating was placed between the CCD camera and microscope objective on the glass window, which was above the tube lens, at a distance of ~ 10.5 cm from the camera chip. The grating and magnification of objectives were chosen to observe maximal separation of fluorescent, absorbed, or scattered light within the first-order spectrum while maintaining both the zero and first-order spectral image within the same field of view. An additional C-mount tube containing a movable slider that held the grating was machined (University of Toledo, Department of Physics Machine Shop). This slider allowed gradual change of the distance between the microscope objective and the CCD camera between 6 cm and 7 cm. However, all images presented in this manuscript were acquired using the 70 grooves/mm grating set at a distance of 10.5 cm from the CCD camera chip. According to manufacturer specification, this grating typically transmits 41% of light into the zero order and 32% of light in the first order ($n = +1$) at 632 nm, while light diffracted into other orders is negligible. To measure grating transmission at other wavelengths, a FocalCheckTM fluorescence test slide was used and regions of interest (ROIs) were imaged around zero and first-order images of fluorescent nanoparticles.

Image Analysis

Images were acquired and analyzed using MS Elements imaging software (Nikon). Intensity profiles were also plotted using this software. Distances between first- and zero-order images were measured and expressed in pixels or micrometers. Because the CCD camera is

monochrome, images acquired with separate filter cubes were pseudo-colored depending on the filter cube used (DAPI: blue, FITC: green, and TRITC or Texas Red: red). A spectral image was analyzed by iterative deconvolution using Image J software (NIH). In the presented image, the monotonic deconvolution option was selected, the number of deconvolutions was set at 2, and the LP filter was set at 0.1 pixels.

Results and Discussion

Spectral Imaging Through a Transmission Diffraction Grating

A transmission diffraction grating disperses the light of various wavelengths (λ) through multiple grooves into spectral orders (n).¹⁹ For example, a transmission diffraction grating used previously for fluorescence spectroscopy of individual cells⁹ and single molecules^{13,14} transmits incident light into a zero-order image ($n = 0$) and diffracts light mostly into the first-order ($n = +1$) spectrum. It can be expected that this diffraction grating will disperse in a similar way the light scattered or transmitted by the cells in darkfield and brightfield or DIC microscopy, respectively. Hence, in spectral imaging mode, light emitted, scattered, or transmitted by the cells will be dispersed by the transmission diffraction grating into the first-order spectral images and transmitted into zero-order images that are equivalent to the images of cells without the grating. Wavelengths in the first-order spectral images can be determined using the grating equation for normal incident light:

$$n\lambda = d \sin \theta \quad (1)$$

where d represents the grating constant (i.e., the spacing between grooves of a grating). To calculate a wavelength in the first-order spectrum, the diffraction angle (θ) should be determined from the following equation:

$$\theta = \tan^{-1}(d_2/d_1) \quad (2)$$

In this equation, d_1 represents the distance between the grating and the CCD camera, and d_2 represents the displacement distance between the zero-order image and the first-order spectral image of the cell.

Description of Multimodal Spectral Imaging Methodology

The schematic diagram shown in Fig. 1 explains how a conventional light microscope is used for multimodal spectral imaging of cells upon incorporation of a transmission diffraction grating. The grating is positioned between the microscope objective and the CCD camera above a fluorescence filter cube and microscope tube lens. In the fluorescence mode, cellular fluorescence is excited by the light of a mercury lamp and collected through a filter cube containing an excitation filter, a dichroic mirror, and an emission filter. For each cell on the slide, the CCD camera records at the same time zero-order images of cells (white dots in Fig. 1) and first-order spectral images corresponding to emission fluorescence spectra of fluorophores that are used to label cells. As expected from the grating equation (Eq. 1), a grating with a higher number of grooves (i.e., lower value of grating constant, d) will spread the first-order image more but will have better spectral resolution than a grating with a lower number of grooves (larger d). Spectral resolution can also be increased if a grating is set further from the CCD camera (larger d_1) or if a CCD camera with smaller pixels is used.

First-order spectral images can be used to determine fluorescence spectra of fluorophores present in cells in the range of wavelengths selected by the emission filter of a fluorescence filter cube. Wavelengths (λ) in the first-order spectrum are calculated using the diffraction grating equation (Eq. 1) upon calculation of diffraction angles corresponding to these wavelengths (Eq. 2). The displacement distance (d_2) between zero-order and first-order images is measured by the camera software in pixels and converted into units of length upon multiplication with the size of a pixel. It is hard to measure precisely the exact distance between the camera and the grating (d_1) because of the unknown thickness of the camera window.⁹ Therefore, the distance between the camera and the grating was calculated by recording the fluorescence signal of a compound with known emission properties (FITC) as described below.

The grating-based spectral microscope can record the spectrum of the light scattered by the cells. To perform this experiment, the fluorescence filter cube (Fig. 1) is rotated out of the light path and cells are selectively illuminated through a colored filter (e.g., green bandpass filter) and a darkfield condenser by a halogen lamp, whose light reaches the cells from the bottom of the slide. First-order spectral images of cells represent light scattered by the cells upon their illumination with the incident light selected by the filter, and corresponding scattering wavelengths can also be calculated using Eqs. 1 and 2.

The setup can also measure the absorption spectra of dye-labeled cells (e.g., hematoxylin and eosin labeled cells) or other colored specimens that are illuminated through a DIC/bright-field condenser. Instead of peaks with higher intensity than the background, as is the case in darkfield and fluorescence imaging, absorption bands that appear in the first-order spectral images will have a lower intensity than the background due to the absorption of incident light by the cells.

Alternatively, unlabeled or fluorescently labeled cells illuminated through a colored filter (e.g., green bandpass filter) and a DIC/brightfield condenser will absorb the color of light selected by a filter, and the first-order spectral images of cells will show up as well. These spectral images are due to selective illumination through a colored filter and higher absorption (i.e., lower transmission) of incident light through cells in comparison to the surrounding medium. Hence, these spectral images do not show up because of absorption of incident light by specific chromophores present in the cells.

Fluorescence Spectral Imaging of Yeast Cells Labeled with Multiple Florescent Dyes

The grating-based spectral microscope can perform spectral differentiation of multiple fluorophores in individual cells and cellular organelles. Figure 2 shows the fluorescence spectral image of a yeast cell (diameter $\sim 5 \mu\text{m}$), labeled with DAPI, FITC, and RITC. These dyes bind to the cell nucleus (DAPI) and cellular proteins (FITC and RITC) and are spectrally distinguished in the yeast cell using the grating and a triple filter cube that contains excitation and emission filters specific for all dyes. The dyes can also be spectrally characterized by the grating when excitation and emission filters specific for each dye are used (see Fig. S-1, in the Supplemental Material, available online), but this experiment takes longer because three filter cubes are used interchangeably instead of one triple filter cube. Hence, the diffraction grating setup in combination with a triple filter cube provides simultaneous spectral imaging of all three dyes in a single experiment. The combination of the grating with filter cubes also distinguishes multiple fluorescent dyes that are localized in the same location (e.g., in a single organelle). For example, FITC and RITC are bound to cellular proteins throughout the cytoplasm of the yeast cell, but they are clearly distinguished by the grating because of differences in their fluorescence emission spectra.

Additionally, the grating-based spectral microscope can be used to characterize fluorophores by the calculations of their emission fluorescence spectra using Eqs. 1 and 2. To accurately determine the distance between the diffraction grating and the camera chip (d_1), the distance between peaks in the intensity profile of the first- and zero-order images of FITC (fluorescence emission maximum at 525 nm) was measured in labeled yeast cells (Fig. 2). This distance was 599 pixels (3864 μm) using a grating with 70 grooves/mm ($d = 1/70 \text{ mm}$). Using the grating equation (Eq. 1), it could be calculated that light with a wavelength of 525 nm is dispersed at an angle of 2.10° . Further, it was derived using Eq. 2 that the distance between the grating and camera was 10.5 cm. This value was used to calculate wavelengths corresponding to fluorescence emission maxima of other dyes. From the intensity profile shown in Fig. 2, it was found that emission maxima of DAPI and RITC are at 456 nm (517 pixels away from zero-order maximum) and 610 nm (696 pixels away from zero-order maximum), respectively. Calculated values closely match previously reported emission maxima of these dyes. Because fluorescence emission maxima of DAPI and RITC are 154 nm and 179 pixels apart, the spectral resolution of the system is $\sim 0.86 \text{ nm/pixel}$. Intensity profiles can also be exported into a spreadsheet and plotted as conventional emission fluorescence spectra (i.e., relative fluorescence intensity versus wavelength) upon appropriate calculations. Figure 3 shows the emission spectrum of a yeast cell labeled with DAPI, FITC, and RITC. To plot this spectrum, the maximum intensity of the zero-order image (Fig. 2) was set as zero of the wavelength scale and wavelengths corresponding to fluorescence spectra of dyes were calculated and plotted along with associated fluorescence intensities.

Fluorescence Spectral Imaging of Fluorescently Labeled Organelles in BPAE Cells

As well as performing spectral differentiation of multiple fluorescent dyes, the grating-based spectral microscope can spectrally image with high resolution dyes bound to various cellular organelles. Figure 4 shows a bovine pulmonary artery endothelial (BPAE) cell whose nucleus (labeled with DAPI), cytoskeleton actin (labeled with Alexa Fluor 488-phalloidin), and mitochondria (labeled with MitoTracker Red) were spectrally and spatially distinguished by the diffraction grating upon illumination through a triple DAPI/FITC/Texas Red filter cube. Therefore, cellular organelles (e.g., nuclei, cytoskeleton, and mitochondria) can be spectrally imaged in BPAE cells based both on their spectral properties and on the morphology of organelles. Alternatively, all three fluorophores can be imaged in BPAE cells using the grating setup along with specific filter cubes (Fig. S-2, panels A–C). The combination of a grating with filter cubes again provides a way to distinguish multiple fluorescent dyes in a single organelle. For example, the nucleus of a BPAE cell shows up twice in the spectral image (Fig. 4) when the grating is used with the triple DAPI/FITC/Texas Red filter cube and it can also be imaged when the grating is used with DAPI and FITC filters separately (Fig. S-2, panels A–C). This is probably due to the binding of the green fluorescent Alexa Fluor 488-phalloidin, which is spectrally distinguished from DAPI, to actin present in the nucleus. Again, displacement distances were measured between maxima of the first-order and zero-order images and used to calculate the wavelengths corresponding to the emission maxima of DAPI, Alexa Fluor 488, and MitoTracker Red. These values were 456 nm, 525 nm, and 604 nm, respectively, and are close to the emission wavelength of each dye reported by the dye manufacturers.

Spectral Imaging of Cells Using Darkfield Microscopy

The grating-based spectral microscope can also record spectral images of cells by darkfield microscopy. In this spectral imaging mode, the grating diffracts light that is scattered by the cells upon their illumination through a darkfield condenser. Figure 5 shows a darkfield microscopy image of a human cheek epithelial cell ($d \sim 50 \mu\text{m}$) and the corresponding first-order spectral image upon illumination of the cell through a green filter ($\lambda = 546 \pm 10 \text{ nm}$).

Using the grating equation (Eq. 1) and intensity profiles in this image, we calculate that cells scatter green light at the maximum wavelength of 543 nm, which matches closely the wavelength of the green bandpass filter used for illumination of cells. This experiment indicates that scattering spectral characteristics of biological cells can be measured using the grating-based setup.

Spectral Imaging of Stained Cells by Brightfield and Differential Interference Contrast Microscopy

Spectral imaging of various chromophores and colored biomarkers is useful for colorimetric investigation of cells and tissues. To test whether the grating-based setup can be used for measurement of absorption spectra of dyes, colored marker dots were drawn on a glass slide and imaged by brightfield and DIC microscopy. Figure S-3 shows the measurement of absorption spectra of a red marker dye by brightfield microscopy with the intensity profile shown at the bottom of the image. The absorption maximum of 490 nm was determined for this red dot from the zero-order and first-order images and the grating equation. This absorption maximum wavelength corresponds to the blue-green region of the electromagnetic spectrum, and its color is complementary to the red color of the dot, supporting the calculation. By imaging the same dot under fluorescent illumination (Fig. S-4), it was found that its emission fluorescence spectrum shows a maximum at 586 nm, indicating that the marker may contain a rhodamine-like fluorophore.

Absorption spectral imaging of cells and tissue sections labeled with common colorimetric dyes such as hematoxylin and eosin (H&E) can also be performed using the diffraction grating-based spectral microscope. While hematoxylin binds to nuclei, eosin binds mostly to intracellular and extracellular proteins. Figure 6 shows a DIC microscopy image of a part of the *Amphiuma* tissue section containing hematoxylin and the corresponding intensity profile in the image that was used to determine the absorption spectrum of the dye (Fig. 7). The wavelengths in the absorption spectrum were calculated considering the point of lowest intensity in the zero-order image as the zero of the wavelength scale. The pixel intensities (I) were converted into transmittance ($T = I/I_0$), taking the intensity of the background as the intensity of light reaching the sample (I_0), and the absorbance values were further calculated ($A = -\log T$). Figure 7 shows that the absorption maximum of hematoxylin is at ~650 nm. This value closely matches the literature value of the absorption maximum of hematoxylin bound to a tissue section,²⁰ Figure S-5 shows another part of an H&E-stained *Amphiuma* liver tissue section indicating that spectral properties of multiple cells across a tissue section can be measured.

The setup can be used for brightfield or DIC imaging of unlabeled cells that show sufficient contrast between cellular features. As mentioned above, spectral images of unlabeled cells can also be recorded by brightfield or DIC microscopy using a colored filter in the light path. Figure S-6 shows DIC spectral images of human cheek epithelial cells taken upon their illumination through a green bandpass filter. However, as explained previously, DIC microscopy in this case does not measure absorbance spectra of chromophores present in this cell but rather the absorption spectrum of a colored filter that was inserted in the illumination path.

Simultaneous Use of Multiple Spectral Imaging Modes for Cell Imaging

As shown in Figs. S-3 and S-4, the grating-based spectral microscope can be used for simultaneous spectral imaging of dye specimens by fluorescence and brightfield microscopy. Simultaneous multimodal imaging of fluorescently labeled cells is possible if cells contain structures, dyes, or particles that have fluorescent properties, scatter the light, and have high absorption coefficients (i.e., if they show up with sufficient contrast in all

spectral imaging modes). However, one should be cautious regarding how data are interpreted because cellular fluorescence can be excited not only by epi-fluorescence but also by darkfield or DIC microscopy. For example, Fig. S-7 shows an overlap between spectral fluorescence images of a FITC and RITC labeled yeast cell with corresponding spectral images acquired by DIC microscopy. The fluorescence signals of two dyes acquired by epi-fluorescence microscopy were pseudo-colored in green and red. They overlapped with a fluorescence spectral image of the cell acquired by DIC microscopy (long streak across other images) and a spectral image of the same cell that was acquired by DIC using a green bandpass filter (monochromatic image next to the FITC image). Therefore, the former spectral image represents the emission fluorescence spectrum of the cell instead of its absorption spectrum.

The sensitivity of a grating-based setup for absorption spectra measurement is lower than in the case of fluorescence spectral imaging. This can be noticed in imaging of fluorescently labeled BPAE cells, where brightfield and DIC microscopy neither visualized these cells nor measured absorption spectra of fluorophores in cells (data not shown). However, a spectral microscope containing a transmission diffraction grating can be used in favorable cases to simultaneously record fluorescence, absorption, and/or scattering spectra of cells. Besides localization of various probes in the cells, information on spectral properties of biomolecules and labels that are used to tag cells can be determined by these experiments.

Spectral Characteristics of the Imaging System

The spectral resolution of the described spectral microscope using a grating with 70 grooves/mm was ~ 0.86 nm/pixel. It is independent of the magnification of the objective used, and the distance between the zero- and first-order spectral images of the cells will be the same when objectives of different magnification are used. However, the objective's magnification and NA will determine the spatial resolution of the spectral images because the size and spatial resolution of the first- and zero-order images are dependent on the objective used. Objectives of higher magnification and numerical aperture are desirable so that the best spatial resolution can be obtained. However, the objective magnification should be carefully selected because large cells that are magnified too much could overlap with their first-order spectra, and this may complicate the spectral analysis.

The presented spectral imaging approach is also useful for imaging of multiple cells that can be spectrally imaged on the slide at once (e.g., Figs. S-5 and S-6). A high cell density on a microscope slide may lead to the overlap of spectral images of cells with zero-order images of neighboring cells. This can be readily avoided by the preparation of slides with appropriate cell density. The bigger challenges are to avoid overlap between the first-order spectra of cellular organelles that are located at adjacent positions in the cell and smearing of spectral images due to out-of-focus light. To address these issues, the deconvolution of spectral images was performed. For example, fluorescently labeled mitochondria in Fig. 4 are located so close to each other that their spectra will overlap and make spectral images extra wide. The iterative deconvolution of this figure by Image J software improved the spectral image of fluorescently labeled BPAE cells (Fig. S-8). While algorithms are available for deconvolution of both monochrome and multicolored images, they are mostly optimized for the deconvolution of fluorescence microscopy images rather than spectral images. Therefore, a potential development of spectral imaging deconvolution software could improve the resolution of spectral images.

Another challenge, which is common for all spectral imaging methods, is distinguishing fluorophores or chromophores that have similar spectral properties and are present at the same location in the cells.²¹ Currently, this can be done only by a combination of spectral

imaging with a mathematical procedure called spectral unmixing,²¹ and the development of such a procedure for the presented spectral imaging method would be very beneficial.

The spectral performance of the present system is also dependent on the quality of the grating used. Using the fluorescence test slide, we found that the ratios of the intensities of the first- and zero-order light diffracted by the grating are 8, 3.5, 1.6, 1.5, and 0.9 for blue, green, orange, red, and deep red fluorescent nanoparticles, respectively. Tuning of the spectral properties of the grating can help increase the sensitivity of the spectral imaging measurement in the fluorescence, scattering, or transmission mode.

Applicability of the Spectral Imaging System

Preceding examples showed that the spectral imaging setup can be used for imaging of standard fluorescently labeled cells, unlabeled cells, and stained cells. To check the performance of the system on more complex specimens, mouse vascular endothelial cells were grown and labeled with DAPI, rhodamine 123 (a dye that labels mitochondria), and RITC. As can be seen in Fig. S-9, fluorescent spectral images of cells containing these three dyes bound to nucleus, mitochondria, and cellular proteins can be readily recorded. The described setup is convenient for spectral characterization of cellular organelles and can potentially be used for quantification of signals coming from different cellular compartments. In the present study, spectral information was acquired through bandpass filters, but it is also possible to acquire fluorescence spectra of multiple fluorophores in a cell using longpass filters.⁹

The present setup is extremely sensitive for fluorescence measurements and it could be used for single-molecule fluorescence spectroscopy of highly fluorescent molecules.¹⁴ The sensitivity of the imaging system for scattered light spectral measurement is also high because of the low background of darkfield images. However, the microscope slides and coverslips used for darkfield spectral imaging should be very clean in order to prevent nonspecific scattering. The sensitivity and signal-to-noise ratio of absorption spectra measurements will depend on the absorption coefficient, thickness, and amount (i.e., concentration) of the dyes present in a specimen. In order to tune the present system for absorption spectra measurement, we have imaged 50 μm particles labeled with a red dye whose absorption maximum is broad and peaks at 524 nm. Figure S-10 shows absorption spectral imaging of these nanoparticles by brightfield microscopy, and the calculations indicate a similar value of their absorption maximum. Additionally, nanoparticles with diameters of 800 nm were stained with hematoxylin and imaged by DIC microscopy (image not shown). The signal-to-noise ratio of the first-order spectral image was not sufficient to precisely measure the absorption maximum, but an image corresponding to the absorption maximum of the dye was recorded. Therefore, the sensitivity of the absorption spectral imaging can be tuned by appropriate choice of an objective or a diffraction grating as well as by the labeling of the specimens with appropriate dyes.

Conclusion

Multimodal spectral imaging of cells was demonstrated by the incorporation of a transmission diffraction grating into a light microscope. Fluorescence, scattering, and absorption spectral images of cells were acquired with the presented spectral imaging methodology. Applications described included fluorescence spectral imaging of yeast and mammalian cells labeled with multiple fluorophores, darkfield spectral imaging of unlabeled human cheek cells, and brightfield and DIC spectral imaging of H&E stained cells, fluorescently labeled cells, dyed particles, and free dyes. The multimodal spectral imaging methodology is readily performed on a conventional light microscope and can be applied for spectral imaging analysis of cells and other specimens.

Future work will strive to overcome the aforementioned challenges and improve the technique so that it can be used more efficiently for simultaneous spectral imaging of higher numbers of fluorophores and chromophores in multiple cellular organelles. Despite the challenges, multimodal spectral imaging is a fast approach for spectral imaging of cells and other specimens. Considering the relatively low cost of a transmission grating, it is also an affordable approach for scientists in need of the acquisition of spectral images. Incorporation of the grating into a conventional light microscope is straightforward and potential users can become proficient quickly in application of the described spectral imaging methodology. The methodology is also open to other commonly used applications of microscopic imaging such as optical sectioning of cells and tissues, live cell imaging, and high-throughput cell analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic diagram of the grating-based spectral microscope. The incorporation of a transmission diffraction grating into the light microscope enables spectral imaging of cells by fluorescence, darkfield, brightfield, and DIC microscopy.

Fig. 2.

First-order spectral image (**top left**) of a single yeast cell (**top right**) labeled with DAPI, FITC, and RITC. Fluorescent dyes were excited simultaneously using a triple filter cube and imaged through a diffraction grating by the fluorescence microscope. Intensity profile across first- and zero-order spectral images is shown at the bottom. A 40 $\times$ microscope objective was used for imaging.

Fig. 3.

Fluorescence emission spectrum of a single yeast cell labeled with DAPI, FITC, and RITC. Intensity profile across first- and zero-order spectral images of the cell was measured, and wavelengths were calculated using Eqs. 1 and 2 considering the zero-order maximum as the zero of the wavelength scale.

Fig. 4. First-order fluorescence spectral image (**left**) of a single BPAE cell (**right**) excited through triple DAPI/FITC/Texas Red filter cube. A 40× microscope objective was used for imaging.

Fig. 5. First-order scattering spectral image (**left**) of a human cheek epithelial cell (**right**) recorded by darkfield microscopy through a diffraction grating. Cells were illuminated through a green bandpass filter and a 40× microscope objective was used for imaging.

Fig. 6. First-order absorption image (**top left**) of a part of an H&E stained *Amphiuma* liver tissue section (**top right**). Image was recorded by DIC microscopy through a diffraction grating using a 10× microscope objective. Intensity profile across the first- and zero-order images is shown at the bottom.

Fig. 7.

Absorption spectrum of hematoxylin stain bound to the *Amphiuma* liver tissue section. Intensity profile was measured across first- and zero-order spectral images in Fig. 6, and wavelengths were calculated using Eqs. 1 and 2 considering the zero-order minimum as the zero of the wavelength scale. Absorbance values were calculated as described in the text.