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Spectral contrast imaging microscopy

Sergey A. Alexandrov^{1,*}, Shikhar Uttam¹, Rajan K. Bista¹, and Yang Liu^{1,2}

¹Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15232, USA

²Department of Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15232, USA

Abstract

We introduce a new technique, spectral contrast imaging microscopy (SCIM), for super-resolution microscopic imaging. Based on a novel contrast mechanism that encodes each local spatial frequency with a corresponding optical wavelength, SCIM provides a real-time high-resolution spectral contrast microscopic image with superior contrast. We show that two microscopic objects, separated by a distance smaller than the diffraction limit of the optical system, can be spatially resolved in the SCIM image as different colors. Results with numerical simulation and experiments using a high-resolution United States Air Force target are presented. The ability of SCIM for imaging biological cells is also demonstrated.

Most imaging microscopy techniques are based on converting object parameters (absorption, scattering, optical density, and birefringence) into the intensity distribution. The fundamental diffraction limit, determined by the NA, limits the imaging resolution by restricting the transmitted range of spatial frequencies. For a long time, scientists have tried to overcome the resolution limit [1], and many different techniques have been proposed to achieve super-resolution, including structured illumination [2] and stimulated emission [3] implemented in fluorescence microscopy, optical nanoscopes [4,5], the synthetic aperture [6–12], and ptychography [13]. But all of these techniques require complicated optical setups, or they are time consuming.

A novel principle, referred to as Fourier domain spectral encoding (FD-SE), has recently been demonstrated to achieve the transmission of spatial information beyond an optical system's diffraction limit [14]. This approach is based on encoding each local spatial-frequency component of an object with a corresponding optical wavelength. An important aspect of this approach is that the bandwidth of the transmitted spatial frequency from an object does not depend on the NA of the optical system. As a result, the information about fine structures, encoded as a corresponding wavelength, can be seen as different colors in the image. But up to now, only very preliminary experimental results using simple, 1D periodic gratinglike samples with low spatial resolution (i.e., macroscopic imaging) have been reported.

In this Letter, we adapt this novel FD-SE approach for real-time, super-resolution imaging of microscopic structures and present a new technique—spectral contrast imaging microscopy (SCIM). We show that this approach can be used to form a high-resolution spectral contrast microscopic image whose spatial resolution is well beyond the diffraction limit of the optical system. We demonstrate the feasibility of SCIM to image objects separated by a distance smaller than the diffraction limit of the optical system using both

numerical simulation and experiments. The superior ability of SCIM for real-time imaging of biological cells is also presented.

In brief, the principle of SCIM is as follows: let us assume that an object consists of structural components at different spatial scales, from large to small (fine). Their spatial structures can be described by various spatial frequencies for a given direction. This assumption holds well for most biological samples that have quasi-fractal structures, including cells and cell aggregations, and tissues [15,16]. The optical imaging system acts as a low-pass filter, and the maximum spatial frequency that can be transmitted through the optical system (or minimum size of the spatially resolved local structures) is limited by its NA.

Let us consider, for example, two local microscopic structures within the sample (a and b in Fig. 1) that cannot be resolved by the given optical imaging system. As shown in Fig. 1, each local structure (a or b) has fine structural characteristics described by the spatial frequencies ν_1 and ν_2 , respectively. At plane wave white-light illumination, the local structures (a and b) contribute to a lower spatial-frequency signal, and the fine structures residing in each local structure contribute to a higher spatial-frequency signal. These spatial-frequency signals are spatially separated in the Fourier plane and cannot be transmitted simultaneously through the limited NA of the optical system. To select the light diffracted by the fine structures with high spatial frequencies that far exceed the diffraction limit of the optical system, the range of the transmitted spatial frequencies can be shifted via changing the illumination angle θ [10] (Fig. 1). Further, the quasi-plane-waves, diffracted at a given direction (restricted by mask F in the Fourier plane), also have different spectral bandwidths (e.g., with dominant wavelengths λ_1 and λ_2 in Fig. 1). Thus, the high-spatial-frequency signal, modulated by a lower spatial-frequency signal, is further modulated spectrally. As a result, the information of these local microscopic structures with their fine structural characteristics, encoded with different spectral wavelengths, will be transmitted through the optical system.

Such spectral-spatial filtering of light diffracted at a given direction results in a super-resolved spectral-contrast color image. The image contrast is produced by the structure of the object, and the spatial frequency of each local structural component is visualized as a corresponding optical wavelength (natural color) in the image, without any numerical processing. For example, the local structure (a) with a specific fine structural characteristic (ν_1) may diffract mostly green light at a given direction, whereas another local component (b) with a different fine structure (ν_2) may diffract mostly red light at the same direction (Fig. 1). As a result, these areas can be clearly seen separately in different colors in the image. For objects with a more complicated structure, the color map of the dominant spatial frequency, which characterizes the dominant structure size will be formed. The 2D dominant spatial frequency ν_s can be calculated as $\nu_s(x, y, \lambda) = n|\sin \theta - \sin \alpha(x, y)|/\lambda$, where n is the refractive index.

To justify the principle of SCIM, we perform both numerical simulation and experiments by imaging three elements of Group 9, the group that has the smallest size on the high-resolution (HR) U.S. Air Force (USAF) target, as shown in Fig. 2(a). Each element consists of two subelements whose sizes are 4.9, 4.4, and 3.9 μm for element 1, 2, and 3, respectively. Separations between subelements in the y -direction are 1.9 and 1.7 μm . Each subelement has a three-bar structure (fine structure) with spatial frequencies of $\nu_1 = 512\text{mm}^{-1}$, $\nu_2 = 575\text{mm}^{-1}$, and $\nu_3 = 645\text{mm}^{-1}$ oriented in both the x and y directions. An imaging system with NA = 0.05 is used for both numerical simulation and experiment. The resolution of such an imaging system for the smallest wavelength (444 nm) is 5.4 μm , so all subelements of Group 9 cannot be resolved by this conventional imaging system.

For numerical simulation, we use the illumination wave with three wavelengths (560, 499, and 444 nm). Both conventional bright-field and SCIM images are formed by numerically transferring the diffracted waves through the same imaging system. For SCIM images, object-dependent spatial-spectral filtering (Fig. 1) is applied. The results show that the conventional imaging system captures the spatial frequencies around the main lobe centered at nearly zero spatial frequency without spatial separation of the different wavelengths for each subelement, and any subelements of Group 9 cannot be resolved [Fig. 2(b)], while SCIM captures the spectrally selected light diffracted from the three-bar structures of each x - or y -oriented subelement at a given direction. As a result, the image of each subelement is formed by a corresponding wavelength, and all three x - or y -oriented subelements of Group 9 are clearly resolved as different colors (or wavelengths) in the image [Figs. 2(c) and 2(d)]. The cross-sectional intensity distributions of the SCIM and conventional images [Figs. 2(e) and 2(f)] further confirm the super-resolution ability of SCIM.

In our experiments, we used a reflection configuration at normal illumination and off-axis collection. The parallel white light wave (Xenon lamp 150 W, Oriel), collected by the objective lens, was filtered in the Fourier plane to capture the required bandwidth of the spatial frequencies and spectral range. This situation is equivalent to tilted illumination and low-resolution optical system in Fig. 1. The images were recorded by a color CCD camera (AxioCam HR, Carl Zeiss).

Figure 3 shows the images of the same elements of Group 9 on HR-USAF target used in the numerical simulation. An excellent agreement between the numerical simulation (Fig. 2) and experimental results (Fig. 3) is evident. All subelements are clearly resolved in the SCIM images as different colors [Figs. 3(c) and 3(d)], but not in the conventional microscopic image [Fig. 3(b)]. The spatial frequency of the three-bar structure (fine structure) of each subelement is presented as different colors in the SCIM image. These color images are formed by natural colors or the corresponding wavelengths, rather than pseudo colors. An increase in spatial frequency corresponds to a blueshifted color in the SCIM image. Besides a superior image contrast, such distinct spectral color provides quantitative measure of spatial frequency of fine structures for each subelement, shown in color bar. Our results demonstrate that even simple structure, like three-bar structure of each subelement, can form clear SCIM images, suggesting its potential for imaging complex biological samples with quasi-fractal structure.

The above example presents the color visualization of a 1D structure with different orientations. For real-time imaging of complex objects as a 2D color map of spatial-frequency distribution, we extend the selected collection solid angle for all azimuthal directions. The spectral range needs to be collected for all azimuthal angles simultaneously, which, at normal illumination, can be implemented using an annular-shaped Fourier mask. Consequently, a 4D filtering (2D spatial and 2D spectral) is applied, and a specific color signature is formed for every local structure of the object.

To demonstrate the potential of SCIM for biological imaging, we image unstained HeLa cells (Fig. 4). The HeLa cells (a type of human cervical cancer cell line) were grown in glass-bottom culture dishes using Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 70% humidified incubator at 37 °C, 5% CO₂. After cells reached approximately 80% confluence, cells were fixed in 70% ethanol and remained in phosphate buffer saline (PBS) solution during imaging.

Figure 4 shows the images of a HeLa cell formed using three different contrast mechanisms, including a bright field [Fig. 4(a)], a phase-contrast [Fig. 4(b)] (both AXIO Observer Z1, Carl Zeiss), and SCIM [Fig. 4(c)]. All microscopic images were formed using the same

objective lens (NA = 0.5). Compared to conventional images [Figs. 4(a) and 4(b)], the SCIM image [Fig. 4(c)] clearly presents the best details of the subcellular structures and superior image contrast. Certain fine structural components within both cell nucleus and cytoplasm, which are not easily seen in bright-field and phase contrast images, are clearly visible in the SCIM image, suggesting that SCIM uses a distinct spectral contrast mechanism from conventional microscopic imaging of unstained cells.

In summary, we introduced a new real-time, super-resolution imaging technique, SCIM. We show that, in contrast to conventional imaging microscopy, SCIM can capture spatial information beyond the diffraction resolution limit of the optical system and form images where unresolved microscopic areas are clearly seen separately as distinct colors with superior image contrast. The SCIM is a simple optical system, and its image can be obtained in real time with a single snapshot using a color camera. Spectral device could further improve the resolving power of this technique. SCIM has a great potential for real-time imaging and quantitative characterization of subcellular structures of label-free cells in their natural state.

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Fig. 1. Principle of spectral contrast imaging (θ is the illumination angle, and α is the scattering angle).

Fig. 2.

Numerical simulation: (a) object (three element of Group 9 of HR-Group target), (b) conventional bright-field image, $NA = 0.05$, (c), (d) SCIM images for (c) y - and (d) x -oriented subelements, $NA = 0.05$, and (e), (f) corresponding cross-sectional intensity distributions for the SCIM images, where each wavelength is represented with the corresponding color: 560nm (yellow), 499nm (green), and 444nm (blue), and for the conventional image (black curve) in the image plane along the y direction.

Fig. 3.

Experimental results: (a) high-resolution ($NA = 0.5$) image of object (Group 9 of the HR-USAFA target); (b) conventional image, $NA = 0.05$; (c), (d) SCIM images ($NA = 0.05$) for (c) y - and (d) x -oriented subelements. The scale bar is $4 \mu\text{m}$. The color bar represents dominant wavelengths (λ) and corresponding spatial frequencies (ν_s) of the three-bar structure for each subelement.

Fig. 4. Microscopic images ($NA = 0.5$) of unstained HeLa cell with three different contrast mechanisms: (a) bright-field image, (b) phase-contrast image, (c) SCIM image. The scale bar is $4\ \mu\text{m}$.