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Hyper-spectral imaging in scanning-confocal-fluorescence microscopy using a novel broadband diffractive optic

Peng Wang [a], Carl G. Ebeling [b], Jordan Gerton [b], Rajesh Menon [a,*]

[a] Department of Electrical and Computer Engineering, University of Utah, Salt Lake City, UT 84112, USA
[b] Department of Physics, University of Utah, Salt Lake City, UT 84112, USA

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ABSTRACT

In this paper, we demonstrate hyper-spectral imaging of fluorescent microspheres in a scanning-confocal-fluorescence microscope by spatially dispersing the spectra using a novel broadband diffractive optic, and applying a nonlinear optimization technique to extract the full-incident spectra. This broadband diffractive optic has a designed optical efficiency of over 90% across the entire visible spectrum. We used this technique to create two-color images of two fluorophores and also extracted their emission spectra with good fidelity. This method can be extended to image both spatially and spectrally overlapping fluorescent samples. Full control in the number of emission spectra and the feasibility of enhanced imaging speed are demonstrated as well.

1. Introduction

Fluorescence microscopy has become the essential scientific tool for both static and dynamic observations of biological structures and reactions due to its specificity and ease of use [1,2]. Furthermore, many fluorophores are highly compatible with biological samples and even allow for the detection of single molecules with excellent signal-to-background contrast [3,4]. Recent discoveries of fluorescent dyes with diverse spectral footprints, which can be specifically attached to target molecules, have advanced applications of simultaneously studying multiple cellular features and molecular interactions [5,6]. In this paper, we report on a new hyper-spectral confocal-fluorescence-microscopy technique that exploits a novel broadband diffractive optical component, which is referred to as a polychromat. The polychromat spatially disperses the emitted light onto an electron multiplying charge-coupled device (EMCCD) camera. By applying a novel implementation of the direct-binary-search (DBS) algorithm, we successfully extract the emission spectra from the dispersed image. We further demonstrate the capability of the proposed technique by numerical simulations on broadband spectrum extraction and linear unmixing of both spatially and spectrally overlapping fluorophores. In addition, the advantages of the utilization of polychromat in fluorescence microscopy are examined via exploring alternative polychromat designs.

In our experiments described in this paper, the imaged sample was composed of two distinct fluorescent dyes deposited onto a glass coverslip. These dyes may be excited either by a single laser or multiple laser beams simultaneously, and the signal is collected in the scanning-confocal mode. The signal, which is a mixture of multiple emission spectra, is then spatially separated by the polychromat and assigned to different regions of an EMCCD camera. By integrating the signal within each region separately, and knowing the position of the excitation spot, we cannot only reconstruct multi-color images simultaneously, but also extract their spectral content. In comparison to a conventional spectrometer, our approach provides two key advantages. First, the optical efficiency of the polychromat can be significantly higher than that of a grating-based optical system. This is particularly important for biological samples where low photon counts in the emission signals are common. Second, the polychromat performs both the functions of spectrum separation and imaging, whereas conventional spectrometers require gratings and additional optics. The polychromat also enables greater freedom in choosing the proper spectral footprint for applications with diverse bandwidth and resolution requirements. Finally, we showcase later that we can use the dispersion of the polychromat to computationally ascertain the incident spectrum, and thereby enable hyper-spectral imaging with a considerably simpler hardware than in conventional spectrometers.

Our experimental setup, which is a modified version of a conventional inverted laser-scanning-confocal microscope, is illustrated in Fig. 1. Two laser sources with wavelengths of 488 nm and 561 nm were collimated, made collinear using a dichroic

* Corresponding author.
E-mail address: rmenon@eng.utah.edu (R. Menon).

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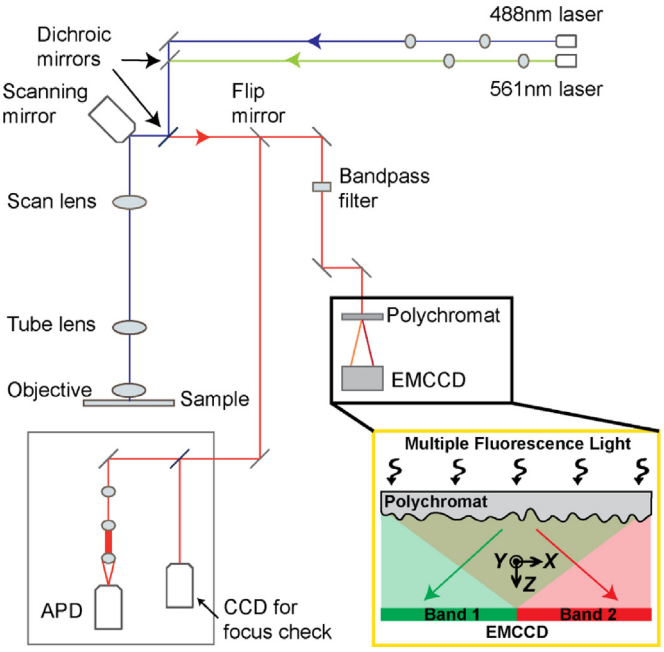

Fig. 1. Schematic of the hyper-spectral scanning-confocal microscope. Two excitation laser lines can simultaneously be focused onto a sample and scanned in a raster fashion. The fluorescence signals are collected and descanned to form collinear collimated beams. Spectrally distinct signals are spatially separated by the polychromat onto different regions of the EMCCD. An APD is used to obtain the control image and a second CCD is used for alignment and checking focus. The hyper-spectral imaging section including the designed polychromat, delineated by the bold black block, is detailed by the schematic in the bold yellow block. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mirror and focused with a single oil-immersion objective onto the sample. The focused excitation spot was quickly scanned across the sample via a mirror-scanning system. As illustrated in Fig. 1, the signal was collected by the same objective (so-called epi-fluorescence configuration) and the same scan mirror "descans" the signal beam. Thereby, at each instant of time, a collimated signal beam is directed towards the EMCCD camera or an avalanche photo-diode (APD). The fluorescence emission spectra are split by the polychromat and are incident on different regions of the EMCCD camera, as illustrated by the inset in Fig. 1.

2. Broadband diffractive optic

The polychromat, as depicted in Fig. 2(a), is a one-dimensional pixelated diffractive optic with quantized heights along one dimension (say, X) [7]. The multi-leveled microstructures are clearly exemplified by the zoom-in plot in the inset. The size in the orthogonal direction was determined by the fabrication constraints as described later. A modified DBS algorithm was utilized to design the pixel-heights of the polychromat such that efficient spatial separation of two distinct spectral bands was achieved in the Fresnel diffraction regime [7,8]. The figure of merit (FOM) for optimization, which was chosen to guarantee numerical convergence is described in Eq. (1). Since the polychromat maintains the same height profile along Y-direction, the numerical simulation and optimization were carried out considering only one-dimension.

$$\mathrm{FOM}=\frac{1}{N}\sum_{i=1}^{N}\varepsilon_i\times\left(\frac{\int_{\lambda^i_{\min}}^{\lambda^i_{\max}}\eta_i(\lambda)d\lambda}{\lambda^i_{\max}-\lambda^i_{\min}}\right). \tag{1}$$

In Eq. (1), i and ε_i are the order and the weighting factor for the ith spectral band, respectively. In this case, $\varepsilon_i=1$ is considered. Each spectral band is delimited by $\lambda^i_{\min}$ and $\lambda^i_{\max}$. N is the total number of spectral bands. In practice, the integral is numerically approximated by the sum over a number of discrete wavelength samples. Spectral efficiency, η_i is defined as:

$$\eta_i(\lambda)=\frac{\int_{x^i_{\min}}^{x^i_{\max}}\mathrm{PSF}(\lambda,x)dx}{\int_{x_{\min}}^{x_{\max}}\mathrm{PSF}(\lambda,x)dx}, \tag{2}$$

in which $x^i_{\min}$ and $x^i_{\max}$ define the spatial region for band i, and $x_{\min}$ and $x_{\max}$ represent the total area of the EMCCD detector. The spatial–spectral point spread function PSF(λ, x) is defined later.

As a proof-of-principle, two spectral bands, with emission peaks at 508 nm and 612 nm, were applied in our polychromat-design model. These peak wavelengths correspond to the specific fluorophores we used in our experiments as described later. The other geometric parameters of the polychromat were: minimum feature size=3 μm; maximum height of each pixel=1.2 μm to render 2π phase shift for the longest wavelength of 700 nm; 64 discrete levels were used for each pixel; propagation distance between the polychromat and the EMCCD plane was 90 mm to satisfy the diffraction condition of the shortest wavelength of 500 nm. The dispersion of the polychromat material, namely a commonly used photoresist, Shipley 1813, was taken into account as well. Periodic boundary condition was applied and one period was comprised of 2000 pixels (total width of 6 mm). The optimization goal was to spatially separate the two spectral bands (500–580 nm for band 1 and 580–700 nm for band 2) onto two adjacent 3 mm-wide areas of the camera. Simulated spectral efficiencies of the optimized polychromat are plotted as solid lines in Fig. 2 (c) and they suggest a spectrally averaged efficiency of above 90%.

Grayscale optical patterning was used to fabricate the polychromat [9]. An array of 3 polychromats were patterned to approximate the periodic boundary condition for the center polychromat. The total patterned area was limited to 30 mm × 30 mm by the exposure tool. A series of calibration exposures were conducted to map the exposure dose to the height topography after development. Our polychromat was fabricated in Shipley 1813 photoresist, which was spun on a RCA-cleaned glass substrate at 3000 rpm and soft baked at 110 °C in an oven for 1 h. This resulted in a photoresist thickness of 1500 nm. After exposure, the photoresist was rinsed in 352 developer for 1 min. An optical micrograph of a part of a polychromat by back illumination is shown in Fig. 2(b). An atomic-force micrograph of a small portion of the polychromat is shown in the inset in Fig. 2(b). This image confirms the multiple height levels.

In order to characterize this polychromat, we illuminated it with a collimated broadband white light from a Xenon lamp. Then, we placed a 50 μm-diameter fiber in its image plane (90 mm away from the polychromat). The fiber was connected to an Ocean Optics spectrometer. The fiber was mounted on a scanning stage, whose axis was orthogonal to the optical axis of the system. The spectrum of the image at each point was obtained and all the spectra were consolidated into a single spatial–spectral plot, which is shown in Fig. 2(e). The corresponding simulation is shown in Fig. 2(d). We define this response as the spatial–spectral point-spread function (PSF) of the polychromat. Note that the agreement between simulations and experiments is excellent. Particularly, the spectral band from 500 nm to 580 nm is located on the left half of the image, while the spectral band from 581 nm to 700 nm is located on the right half. The two bands are well separated with almost no cross talk in the simulated image. The experimental image shows some cross talk, which is likely due to the presence of fabrication errors, deviation from the ideal image distance and errors in the dispersion measurement of Shipley 1813 photoresist.