Multiplexed structured illumination microscopy for simultaneous, sub-diffraction resolution fluorescent and quantitative-phase imaging

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Abstract: We introduce a variant technique to structured illumination microscopy (SIM) that multiplexes fluorescent and quantitative-phase imaging for simultaneous, sub-diffraction resolution, multimodal imaging. Preliminary experimental confirmation is shown for diffraction-limited multi-modal imaging.
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1. Introduction

In the biological sciences, there is much emphasis on high resolution imaging as a means of elucidating biological activities occurring at molecular levels. In some cases, designing optical systems to optimize for aberrations and resolution to achieve diffraction-limited performance is sufficient. In other cases, where still better resolution is required, techniques to achieve sub-diffraction resolution information are necessary. Many sub-diffraction resolution imaging techniques have risen to meet this need, and all have made large impacts in microscopy.

Such sub-diffraction resolution imaging techniques can be roughly categorized into two classes. The first class, often referred to as synthetic aperture, is targeted towards breaking the diffraction limit when imaging a sample with coherent light. Synthetic aperture techniques often rely on tilting the illumination beam at various angles at the sample, thus diffracting different diffraction-limited regions of the sample's frequency spectrum into the imaging system's aperture. The final reconstructed image will have a total frequency support synthesized from all the individual diffraction-limited regions, and will thus offer sub-diffraction resolution [1,2].

The second class of sub-diffraction resolution imaging is targeted towards fluorescent samples, and is often referred to as "super-resolution." This class of "super-resolution" techniques hinges on obtaining sub-diffraction resolution imaging by utilizing properties of fluorophores. This is done by either localizing individual fluorescent emitters to sub-diffraction levels to build a "map" of fluorophore positions, as is done in single-molecule detection techniques such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), or by using spatially modulated excitation to effectively shape the optical PSF, as is done in stimulated emission depletion (STED), ground state depletion (GSD), and structured illumination microscopy (SIM) [3,4].

We have recently introduced structured illumination diffraction phase microscopy (SI-DPM) as a variant of SIM that works via synthetic aperture to allow sub-diffraction resolution, quantitative-phase (QP) imaging of transparent samples, specifically cells [5]. However, although QP imaging allows easy imaging of cellular structure with endogenous contrast via quantitative information inaccessible by conventional fluorescence imaging (such as refractive index or path length), QP imaging inherently lacks molecular specificity. Because the optical setup for SI-DPM is similar to that of SIM, there is potential for combining SI-DPM with SIM for dual QP/fluorescent imaging. Here, we propose a novel system that uses fundamental concepts from SI-DPM and SIM to multiplex QP and fluorescent information onto the camera for sub-diffraction resolution, simultaneous QP/fluorescence imaging.

2. Theory

We divide our theory section into two parts. In the first part, we describe our novel mechanism that allows simultaneous, multiplexed QP/fluorescent acquisition with just one camera. In the second part, we describe how this can be incorporated with SIM concepts for sub-diffraction resolutions.

2.1. Simultaneous quantitative phase and fluorescence imaging

We first review the basics of phase reconstruction by off-axis digital holography, as the groundwork of our quantitative-phase imaging technique. Consider the sample function, given by $x(\boldsymbol{r})$, under some illumination field pattern, given by $i_p(\boldsymbol{r})$, to form a coherent image at the camera, given by $y_p(\boldsymbol{r})$. Here, $\boldsymbol{r}$ is the 2D spatial coordinate vector. This coherent image is then interfered by an off-axis reference wave, given by $U_r(\boldsymbol{r}) = \exp(-j\,\boldsymbol{k_p}\cdot\boldsymbol{r})$, where $\boldsymbol{k_p}$ is defined as a direction vector. Thus, the net interference pattern at the camera from coherent diffraction is given by:

$$n_p(\boldsymbol{r}) = \left|y_p(\boldsymbol{r}) + U_r(\boldsymbol{r})\right|^2 = 1 + \left|y_p(\boldsymbol{r})\right|^2 + y_p(\boldsymbol{r})\exp\left(-j\,\boldsymbol{k_p}\cdot\boldsymbol{r}\right) + {y_p}^*(\boldsymbol{r})\exp\left(j\;\boldsymbol{k_p}\cdot\boldsymbol{r}\right) \quad (1)$$

Now, consider that the sample can also form a fluorescent image at the camera, given by $y_f(\boldsymbol{r})$. If we modulate this image with a carrier wave $c(\boldsymbol{r}) = 1 + \cos\left(\boldsymbol{k_f}\cdot\boldsymbol{r}\right)$, where $\boldsymbol{k_f}$ is another direction vector, then the net fluorescence detected at the camera is given by:

$$n_f(\boldsymbol{r}) = c(\boldsymbol{r})\cdot y_f(\boldsymbol{r}) = \left[1 + \cos\left(\boldsymbol{k_f}\cdot\boldsymbol{r}\right)\right]\cdot y_f(\boldsymbol{r}) = y_f(\boldsymbol{r}) + y_f(\boldsymbol{r})\exp\left(-j\,\boldsymbol{k_f}\cdot\boldsymbol{r}\right) + y_f^*(\boldsymbol{r})\exp\left(j\;\boldsymbol{k_f}\cdot\boldsymbol{r}\right) \quad (2)$$

The coherent diffraction and fluorescent emission incoherently add at the camera so that the camera's net raw acquisition is given by

$$\begin{aligned} n_T(\boldsymbol{r}) = {} & n_p(\boldsymbol{r}) + n_f(\boldsymbol{r}) \\ & = 1 + \left|y_p(\boldsymbol{r})\right|^2 + y_f(\boldsymbol{r}) + y_p(\boldsymbol{r})\exp\left(-j\,\boldsymbol{k_p}\cdot\boldsymbol{r}\right) + {y_p}^*(\boldsymbol{r})\exp\left(j\;\boldsymbol{k_p}\cdot\boldsymbol{r}\right) \\ & \quad + y_f(\boldsymbol{r})\exp\left(-j\,\boldsymbol{k_f}\cdot\boldsymbol{r}\right) + y_f^*(\boldsymbol{r})\exp\left(j\;\boldsymbol{k_f}\cdot\boldsymbol{r}\right) \end{aligned} \quad (3)$$

In Fourier space, terms 4-5 and 6-7 frequency shift copies of the sample's coherent and fluorescent spectra, respectively, away from the frequency support of the first three "ambiguity" terms which are DC centered. If these shifts are sufficient (requiring proper design of the optical system) to separate the spectra copies completely from the ambiguity terms as well as themselves, standard Fourier post-processing steps can be used to reconstruct QP and fluorescence images from $n_T(\boldsymbol{r})$. We emphasize that these QP and fluorescent images are processed out of a single raw acquisition, and are thus acquired simultaneously. If interested in only diffraction-limited imaging, this is the basis of a 1-shot, true-simultaneous QP/fluorescent imaging technique that does not require multiple cameras, ports, or toggle filters to acquire both QP and fluorescent images.

2.2. Sub-diffraction resolution via structured illumination

From imaging theory, the coherent and fluorescent images reconstructed from a single raw acquisition are given by $y_p(\boldsymbol{r}) = h_c(\boldsymbol{r}) \otimes \left[x_p(\boldsymbol{r})\cdot\left[h_c(\boldsymbol{r}) \otimes i_p(\boldsymbol{r})\right]\right]$ and $y_f(\boldsymbol{r}) = h_{ic}(\boldsymbol{r}) \otimes \left[x_f(\boldsymbol{r})\cdot\left[h_{ic}(\boldsymbol{r}) \otimes i_f(\boldsymbol{r})\right]\right]$, respectively. Here, $\otimes$ is the convolution operator, $h_c(\boldsymbol{r})$ and $h_f(\boldsymbol{r})$ are the system's coherent and incoherent point spread functions, respectively, and $i_p(\boldsymbol{r})$ and $i_f(\boldsymbol{r})$ are the field and intensity illumination structured patterns at the sample. We also note from imaging theory that $h_{ic}(\boldsymbol{r})$ has twice the frequency support as $h_c(\boldsymbol{r})$ and $i_f(\boldsymbol{r}) = \left|i_p(\boldsymbol{r})\right|^2$ [6]. In Fourier domain, the transforms of $y_p(\boldsymbol{r})$ and $y_f(\boldsymbol{r})$ are given by:

$$Y_p(\boldsymbol{\omega}) = H_c(\boldsymbol{\omega})\cdot\left[X_p(\boldsymbol{\omega}) \otimes H_c(\boldsymbol{\omega})\cdot I_p(\boldsymbol{\omega})\right] \text{ and } Y_f(\boldsymbol{\omega}) = H_{ic}(\boldsymbol{\omega})\cdot\left[X_f(\boldsymbol{\omega}) \otimes H_{ic}(\boldsymbol{\omega})\cdot I_f(\boldsymbol{\omega})\right] \quad (4)$$

where $Y_p(\boldsymbol{\omega}), H_c(\boldsymbol{\omega}), X_p(\boldsymbol{\omega}),$ and $I_p(\boldsymbol{\omega})$ are the Fourier transforms of $y_p(\boldsymbol{r}), h_c(\boldsymbol{r}), x_p(\boldsymbol{r}),$ and $i_p(\boldsymbol{r})$ respectively and $Y_f(\boldsymbol{\omega}), H_{ic}(\boldsymbol{\omega}), X_f(\boldsymbol{\omega}),$ and $I_f(\boldsymbol{\omega})$ are the Fourier transforms of $y_f(\boldsymbol{r}), h_{ic}(\boldsymbol{r}), x_f(\boldsymbol{r}),$ and $i_f(\boldsymbol{r})$ respectively. $H_c(\boldsymbol{\omega})$ and $H_{ic}(\boldsymbol{\omega})$ are the system's transfer functions for coherent and incoherent imaging with bandwidths of ω_c and $2\omega_c$, respectively. Under uniform illumination, $i_p(\boldsymbol{r}) = i_f(\boldsymbol{r}) = 1$ and thus $I_p(\boldsymbol{\omega}) = I_f(\boldsymbol{\omega}) = \delta(\boldsymbol{\omega})$. This leads to the diffraction-limited coherent and fluorescent spectra given by the following low-pass equations:

$$Y_{DL,p}(\boldsymbol{\omega}) = H_c(\boldsymbol{\omega})\cdot X_p(\boldsymbol{\omega}) \text{ and } Y_{DL,f}(\boldsymbol{\omega}) = H_{ic}(\boldsymbol{\omega})\cdot X_f(\boldsymbol{\omega}) \quad (5)$$

Two interfering beams generate the structured patterns $i_p(\boldsymbol{r}) = \cos(\boldsymbol{\omega_0}\cdot\boldsymbol{r} + \phi_n)$ and $i_f(\boldsymbol{r}) = 1 + 1/2\,\cos(2\,\boldsymbol{\omega_0}\cdot\boldsymbol{r} + 2\phi_n)$, such that $|\boldsymbol{\omega_0}| \le \omega_c$. Fourier transforming and plugging into Eq. 5 above, we get the expressions describing the raw coherent and fluorescent acquisitions under structured illumination:

$$\begin{aligned} Y_{SI,p}(\boldsymbol{\omega}) &= H_c(\boldsymbol{\omega})\cdot\left[X_p(\boldsymbol{\omega}-\boldsymbol{\omega_0})\,e^{-j\,\phi_n} + X_p(\boldsymbol{\omega}+\boldsymbol{\omega_0})\,e^{j\,\phi_n}\right] \text{ and} \\ Y_{SI,f}(\boldsymbol{\omega}) &= H_{ic}(\boldsymbol{\omega})\cdot\left[X_f(\boldsymbol{\omega}) + \frac{1}{2}X_f(\boldsymbol{\omega}-2\boldsymbol{\omega_0})\,e^{-j\,2\phi_n} + \frac{1}{2}X_f(\boldsymbol{\omega}+2\boldsymbol{\omega_0})\,e^{j\,2\phi_n}\right] \end{aligned} \quad (6)$$

As seen, $Y_{SI,f}(\boldsymbol{\omega})$ and $Y_{SI,p}(\boldsymbol{\omega})$ contain sample diffraction and fluorescent information downshifted from higher frequency regions into the system's passband. Using standard SIM processing steps, these frequency regions can be

demodulated back in Fourier space for final image reconstructions up to twice the diffraction-limit for both QP and fluorescent imaging.

3. System Design and Results:

To show experimental verification of this theory, we build the optical system shown in Fig 1a below. A 410±20 nm broadband illumination beam (NKT Photonics, EXW-6) is collimated and transmitted through a Ronchi grating (DG, Edmund Optics 100 lpmm). This grating is imaged via a 4f-system (L1 → OBJ), with a 0 order block, onto the sample, serving as the structured illumination pattern. The coherent diffraction from the sample is sent through a diffraction phase setup [5,7] where a common-path configuration uses a grating (DG1, Edmund Optics 150 lpmm), spatial filter, and the broadband illumination to achieve off-axis interference with high temporal phase stability and low coherent noise. The fluorescence from the sample is split off from the coherent diffraction with a dichroic mirror (DM) and imaged onto another grating (DG2, Edmund Optics 150 lpmm). The fluorescence is optically heterodyned with DG2's profile and imaged via 4f-system (L3 → CMOS) to the camera. We orient DG1 and DG2 orthogonally so that their corresponding carrier waves maximize the separation between the coherent-diffraction and fluorescence frequency components to allow for easy digital filtering and post-processing reconstruction of QP and fluorescent images. A 10X objective (OBJ, Newport M-10X) was used to set the limiting aperture to a modest diffraction limit (appropriate for demonstration purposes).

For experimental results below, we operate this system at diffraction-limited performance to demonstrate simultaneous QP/fluorescent imaging. We image 10 um fluorescent beads with emission centered at 532 nm. In Figure 1(b-e) below, we show a raw acquired interferogram, with zooms on the orthogonal coherent-diffraction and fluorescent interferences from heterodyning with DG1 and DG2, its associated Fourier spectrum, and the final diffraction-limited fluorescent and QP reconstructions via simple digital filtering of the Fourier spectrum.

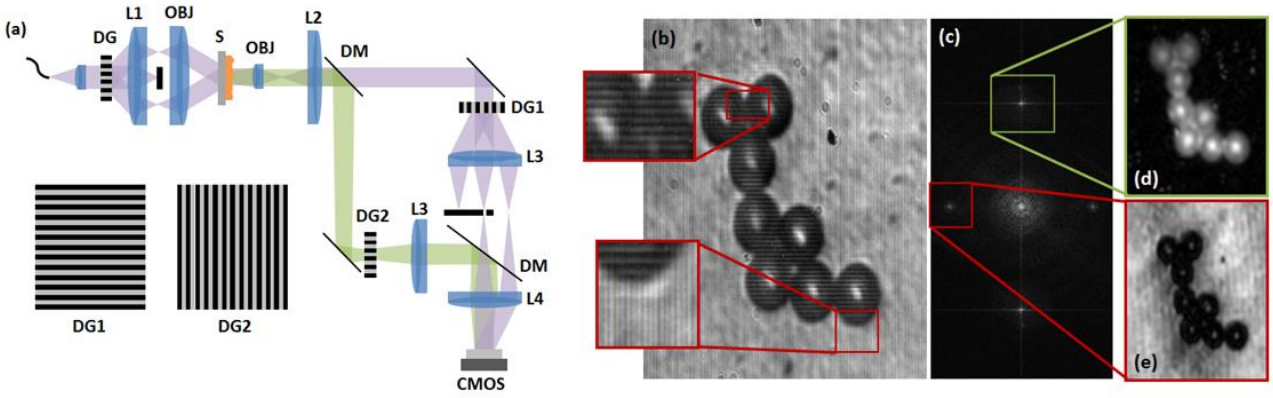

Figure 1 (a) Multiplexed structured illumination QP/fluorescent imaging system. We orient DG1 in the orthogonal direction as DG2. (b) Raw interferogram with zooms highlighting the interference carrier fringes from DG1 and DG2, and (c) associated Fourier spectrum are shown. Digitally filtering select regions of the Fourier spectrum yield (d) fluorescent and (e) quantitative phase images.

4. Conclusions and Future Work

We have introduced a framework that extends SIM to multiplexed QP/fluorescence imaging for simultaneous, multimodal imaging at sub-diffraction resolutions. We experimentally demonstrated the validity of our framework for multiplexed imaging at diffraction-limited resolutions. We are currently working towards experimentally validating this concept at sub-diffraction resolutions via structured illumination.

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5. References

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