Multibeam interferometric illumination as the primary source of resolution in optical microscopy

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High-resolution images of a fluorescent target were obtained using a low-resolution optical detector by illuminating the target with interference patterns produced with 31 coherent beams. The beams were arranged in a cone with 78° half angle to produce illumination patterns consistent with a numerical aperture of 0.98. High-resolution images were constructed from low-resolution images taken with 930 different illumination patterns. Results for optical detectors with numerical apertures of 0.1 and 0.2 were similar, demonstrating that the resolution is primarily determined by the illuminator and not by the low-resolution detector. Furthermore, the long working distance, large depth of field, and large field of view of the low-resolution detector are preserved. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2192153]

Resolution in optical microscopy is often defined in terms of the Rayleigh criterion[1]

$$\delta = 0.61\frac{\lambda}{\mathrm{NA}}, \tag{1}$$

where λ is the wavelength of the light and NA is the numerical aperture (NA) of the objective. Two incoherent point sources are barely resolved by a diffraction-limited system when they are separated by the distance δ. If λ is fixed, resolution can only be increased by increasing NA, which has a maximum value of 1 in air.

Attaining resolution beyond the limit of Eq. (1) is the topic of active research in fluorescence microscopy. One approach that has proven to be effective is the use of structured illumination combined with suitable post-processing methods.[2–7] When the target is illuminated by a series of high-resolution light patterns, high spatial frequency components of the target that are not accessible through the objective can be encoded into low spatial frequencies. Theoretical aspects of this concept have been extensively studied.[8–14]

Previously, structured illumination has been produced by placing a transmission grating at the secondary image plane of the objective[6] or by the interference of a few coherent beams.[7] The main benefit of using structured illumination so far has been *improving* the resolution of a high NA objective. Here we show that when sufficiently many coherent beams produce the structured illumination, it not only improves resolution but can also become a primary mechanism of image formation. In fact, when a total of N coherent beams is used to generate a series of structured illumination patterns, up to $N(N-1)/2$ Fourier transform coefficients of the target can be obtained from brightness measurements from a single pixel.[12–14] The resolution of the reconstructed image is primarily determined by the set of spatial frequencies contained in the series of illuminations, not by the NA of the objective on the detection side.

We generate multiple diffracted beams from a single Ar-ion source (Coherent, Santa Clara, CA) with 488 nm wavelength using an acousto-optic deflector (AOD, Isomet, Springfield, VA, Fig. 1). When the AOD is driven by a radio frequency electrical signal with 31 distinct frequency components (as shown in the frequency analysis above the AOD in Fig. 1), it generates a fan of 31 diffracted beams (as shown to the right of the AOD). The amplitude and phase of each diffracted beam are controlled by changing the amplitude and phase of the corresponding ratio frequency component in the drive signal.[15] An assembly of mirrors then converts the array of diffracted beams into a converging cone of beams, which overlap and form an interference pattern at the target region. Light fluoresced from the target passes through a microscope objective (Carl Zeiss, Germany, 10× with NA of 0.2) and a long-pass filter (CVI Laser, Albuquerque, NM) and is projected onto a charge coupled device (CCD) (Dalsa, Colorado Springs, CO).

Modulating the amplitudes of the beams to turn on a series of 62 different pairs of beams generated a series of illumination patterns, each of which is a sinusoidal brightness grating represented by a single spatial frequency. For each pair of beams, modulating the phase of the second beam at 15 equally spaced phases from 0 to 2π translated the illumination pattern over its single spatial period. The resulting 930 illumination patterns, which are represented by a set of 62 spatial frequencies, were used for a target made of fluorescent beads with 1 μm diameter (Invitrogen, Carlsbad, CA). For each illumination pattern, an image was acquired, resulting in a data set made of 930 images. To show the effect of the NA of objective on resolution, the effective NA was changed from 0.2 to 0.1 by constricting the diameter of the entrance pupil of the same objective and another data set was acquired.[16]

The same data for each NA were processed in two different ways. In the first case, 930 images were averaged to produce a single image, without utilizing the knowledge of the illumination patterns. The averaged image is similar to an image acquired with uniform illumination as in conventional microscopy but has a significantly increased signal-to-noise

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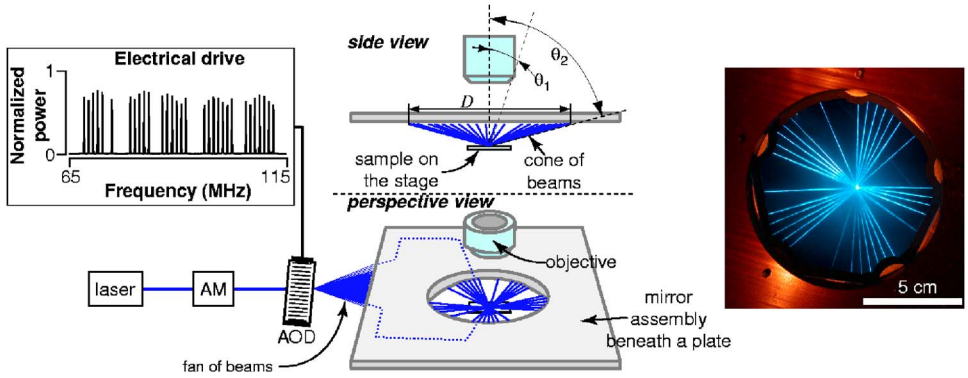

FIG. 1. Schematic of the setup. The side view (upper center) of the setup shows the cone of beams beneath the aluminum plate with half angle of θ_2 (=78°). The cone half angle of the objective θ_1 (=11°) is also indicated. The cone of beams has a diameter D. The panel on the right side is a photograph of the setup looking from the top (at a slight angle) and shows 31 beams visualized through the opening of the aluminum plate (a view from the objective). The bright spot near the center is where the interference pattern forms and the target is placed.

ratio (SNR) compared to a single exposure image due to the averaging. Figures 2(a) and 2(b) show images of the same region of the target generated by averaging 930 images for the NAs of 0.2 and 0.1, respectively. In both images, individual beads in a cluster of beads were not resolved (for example, see cluster inside the box). Also, the decrease in

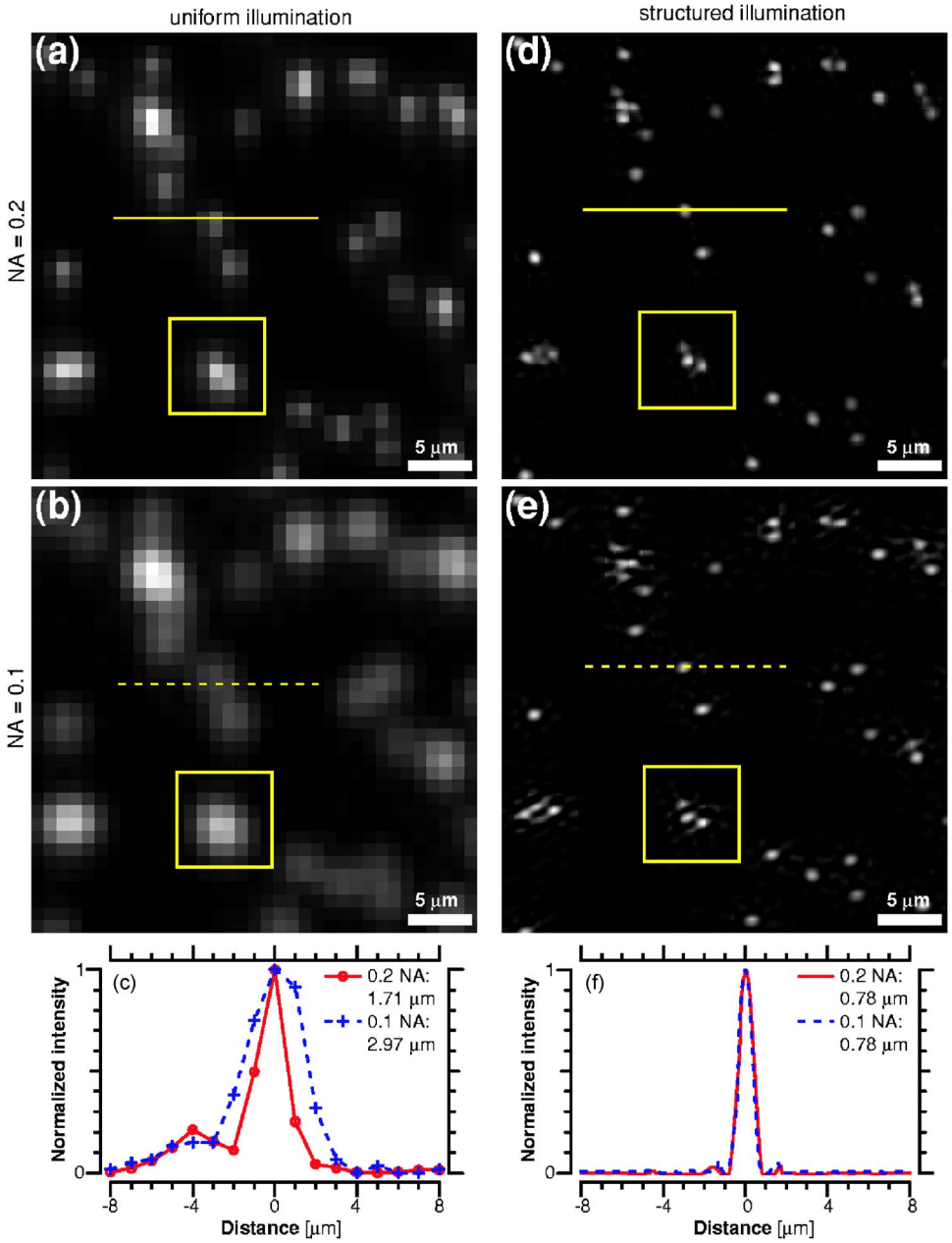

FIG. 2. Effect of NA on resolution in conventional microscopy (uniform illumination, left column) and in multibeam interferometric illumination method (structured illumination, right column). The box in each image contains a cluster of three beads. The plots on the bottom show intensity scans through the center of a bead (along the line in each image) for 0.2 NA (solid line) and for 0.1 NA (dashed line). Estimated FWHM intensities of the bead for each case are indicated.

NA significantly increased blurring; the full width at half maximum (FWHM) intensities of a single bead increased from 1.71 to 2.97 μm when the NA changed from 0.2 to 0.1 [Fig. 2(c)].

In the second case, the data were processed on a per pixel basis; time sequences of 930 brightnesses from each pixel of the CCD were processed to extract 62 Fourier transform coefficients of the target *within* the pixel at 62 spatial frequencies contained in the illumination series. An image of the small region of the target corresponding to the pixel area was then reconstructed using a method similar to inverse Fourier transform.[13] Images independently reconstructed from adjacent pixels were then stitched together to generate an image corresponding to a larger area [Figs. 2(d) and 2(e)].

For each NA of the objective, the resolution of the image reconstructed from structured illumination was higher than that for the uniform illumination case. Notice, for example, that individual beads in a cluster were readily resolved in structured illumination. The objective, the camera, the exposure time, and the excitation light energy are the same in this comparison. Furthermore, changing the NA from 0.2 to 0.1 did not change the width of the bead in the images reconstructed from structured illumination [Fig. 2(f)] even though it increased the width using uniform illumination by a factor of 1.74 [Fig. 2(c)]. These results demonstrate that the resolution of the images in Figs. 2(d) and 2(e) derives from the structured illumination, not from the objective.

In conventional light microscopy, the physics of lenses impose relations between resolution and other optical parameters of the system. For example, the numerical aperture of a lens cannot be increased to improve resolution without either increasing the size of the lens or decreasing the working distance. The technique presented here removes many of these constraints by decoupling resolution from other optical parameters. In particular, the resolution is primarily determined by the illumination, while the working distance, depth of field, and field of view are primarily determined by the objective. Thus the working distance, depth of field, and field of view are significantly greater than can be achieved without greatly increasing the size of the lens or reducing the resolution of the images.

Furthermore, the resolution of the structured illumination can be increased beyond what is possible with visible wavelengths if one can scale the illumination wavelength to ultraviolet and even x ray. This is because the apparatus that produces it could be made entirely of mirrors (reflective elements). Such resolution improvement through wavelength scaling is significantly more challenging with a lens (a refractive element), since high numerical-aperature lenses are difficult to construct at such short wavelengths of light.

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[15] The optical phase relationship of the diffracted beams set by the electrical drive is not maintained over time due to the Doppler shift effect. This is compensated by properly choosing a set of drive frequencies and by stroboscopically gating the source beam with the amplitude modulator (AM in Fig. 1).
[16] The CCD exposure times for each NA were 40 ms (0.2 NA) and 600 ms (0.1 NA).