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Simultaneous two color image capture for sub-diffraction localization fluorescence microscopy

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ABSTRACT

A sub-diffraction limit fluorescence localization microscope was constructed using a standard cooled 1.4 mega-pixel fluorescence charge-coupled device (CCD) camera to simultaneously resolve closely adjacent paired quantum dots on a flat surface with emissions of 540 and 630 nm. The images of the overlapping Airy discs were analyzed to determine the center of the point spread function after noise reduction using Fourier transformation analysis. The Cartesian coordinates of the centers of the point spread functions were compared in serial images. Histograms constructed from serial images fit well to Gaussian functions for resolving two quantum dots separated by as little as 10 nm in the *x*–*y* coordinates. Statistical analysis of multiple pairs validated discrimination of inter-fluorophore distances that vary by 10 nm. The method is simple and developed for *x*–*y* resolution of dilute fluorophores on a flat surface, not serial *z* sectioning.

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1. Introduction

Advances in fluorescence microscopy have enabled resolution of distances beneath the light diffraction limit. After confocal laser scanning microscopy (Cremer and Cremer, 1978), resolution improved with illumination techniques that exploited evanescence of light in total internal reflectance fluorescence (TIRF) (Axelrod, 1981), and scanning near field optical microscopy (SNOM) (Betzig et al., 1991). Standing light waves were used in structured illumination microscopy (SIM) (Bailey et al., 1993). Non-linear responses of fluorophores have been harnessed in other techniques such as stimulated emission depletion microscopy (STED) (Hell and Wichmann, 1994).

Methods that use the point spread function of fluorescence emission to position emitters are referred to as localization microscopy. For photons emitted from the same source, the center of the point spread function reflects the photon probability distribution and is estimated more accurately than the width of the beam that is determined by photon position (Agard and Sedat, 1983). Variations of these techniques allow sequential isolation of emissions separated in time and space (e.g., confocal microscopy (Bornfleth et al., 1998), photoactivated localization microscopy (PALM) (Betzig et al., 2006), fluorescence photoactivation localization microscopy (FPALM) (Hess Girirajan and Mason, 2006; Hess et al., 2009), stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006). A comparison of techniques shows an approximate resolution in the *x*–*y* plane in images of about 20 nm (Schermelleh et al., 2010). Generally, the instruments to achieve "super-resolution" are expensive and remain out of the reach of the average researcher. Yet many applications require only the determination of distance between fluorophores in one plane. Because of the pioneering work in many disciplines, resolution predicated only on the Gaussian fit of the point spread function and the number of photons detected can be hypothetically realized with inexpensive lasers, robust single point emitters (e.g., quantum dots), CCD cameras and commonplace computer software. Noise reduction is now routine by Fourier transformation and can further improve the image quality. Use of simultaneous two color image capture and discrimination of different fluorophores potentially could eliminate the effects of motion and obviate the need for registration or fiducials for images collected separated either in time or space. With the goal of simplicity we assembled an uncomplicated microscope and camera of modest resolution to test nanoscale precision potentially accessible by virtually all laboratories.

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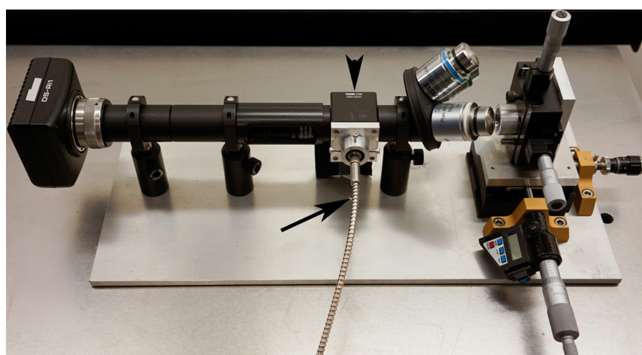

Fig. 1. Microscope for simultaneous 2 color capture localization is shown on an air table. From the left a black CCD camera is connected to a tube containing a focusing lens. The dichroic mirror and filters (arrowhead) are attached to the multimode fiber laser input (arrow). The objects and stage are shown at the far right.

2. Materials and methods

2.1. Microscope

The home built microscope is shown in Fig. 1. The excitation source, a 405 nm, 150 mW, diode laser (Thorlabs, Inc.), 3.8 mm beam diameter, is driven by a 250 mA blue laser diode drive board (Thorlabs, Inc.). Transmitted light is focused into a multimode fiber optic patch cable (Thorlabs, Inc.) and the beam is reflected with a 409 nm 25 × 36 mm Bright line single edge dichroic (Semrock, Inc.) and focused with a 1.4 numerical aperture 100× objective lens (Carl Zeiss, A.G., 440780-9904). Glass slides are mounted to the stage on a hollow aluminum cylinder that is secured with a through bolt to minimize motion. The samples may be viewed from above or from the side by shifting the position of the stage. Stage movement is facilitated with 3 axis adjustment micrometers (Mitutoyo Corp and Newport Corp.). The focal length is adjusted with a differential actuator (1/2" manual drive with 0.5 μM graduated lockable thumbscrews (Thorlabs, Inc.). Emitted light passes through a 500 nm cutoff long pass filter FEH 0500 (Thorlabs, Inc.) enabling red and green to be visualized simultaneously. Emitted light is captured with 1.4 MP Nikon DS-Ri1 camera, Peltier cooled to −10°. Exposure time at 80 ms provides optimal image quality while preserving sample integrity. The diode laser is controlled and synchronized to the corresponding exposure period. The pixel size is 55 nm.

2.2. Samples preparation

Six nanometer diameter CdSeS/ZnS alloyed quantum dots (Sigma–Aldrich Co.) with emission maxima of 540 and 630 μm were diluted 1:2500 in a solution of toluene and 3 μl were dried on a 22 × 22 mm cover glass of 170 μm thickness. Multiple frames of the same field were recorded and later analyzed to spatially resolve overlapping quantum dots of different colors.

2.3. Image analysis

Raw images were recorded as 16 bit RGB tiff files and analyzed in Matlab (MathWorks, Inc.) according to the algorithm in Fig. 2. The program was instructed to separate bright spots from background using global gray scale threshold regions, which correspond to the Airy discs of closely paired red and green fluorophores. The regions of interest around the Airy discs were restricted to the presence of both green and red colors in the bright spots of the original image. Noise reduction was performed on the designated spots by Fourier transform (Fig 3). A filter mask was placed around the central area of the Fourier transformed image to eliminate high

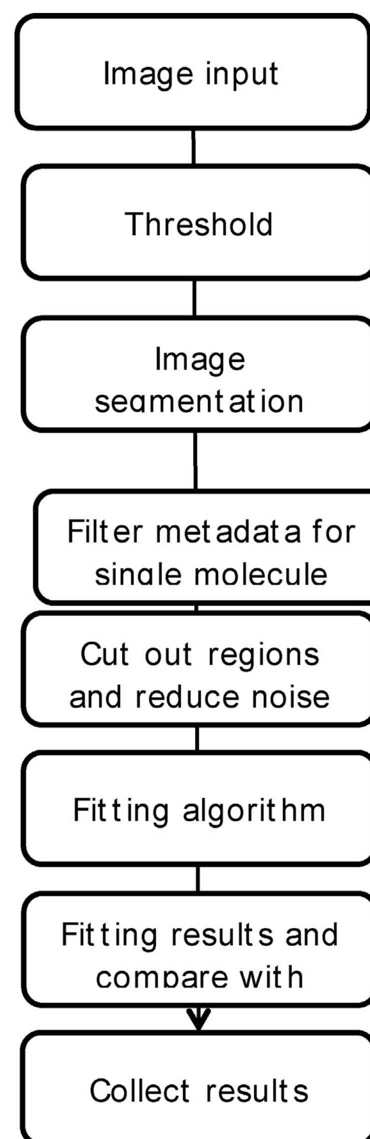

Fig. 2. Algorithm for image analysis from top to bottom.

frequency noise. Finally, an inverse Fourier transform recovered the real image from Fourier space. The centers of the point spread function of the paired fluorophores were transposed to Cartesian coordinates using the same frame of reference and the program was instructed to calculate the inter-fluorophore distances for all green and red quantum pairs in each image. The de-noised intensity data from red and green color channels were then fitted to a two dimensional Gaussian formula by least-squares analysis:

$$f(x,y) = A\exp(-((x-x_o)^2/2\sigma^2) + (y-y_0)^2/2\sigma^{2)}) + z_o \quad \text{(A1)}$$

where A is the amplitude or intensity, x_o,y_o is the center, σ is the standard deviation, z_o is an offset constant.

In order to evaluate the statistical properties of calculated distances between quantum dots for each pair set, histograms of pair distances were fit to a standard Gaussian equation for analysis:

$$f(x) = A\exp(-((x-{x_o}^2/2\sigma^2) + z_o \text{withthesameparametersas(A1).(A2)}$$

The coefficient of determination (R2) was calculated:

$$\text{R2} = 1 - \text{residualsumofsquares/totalsumofsquares.} \quad \text{(A3)}$$

To assess the fidelity with which the method discriminates various inter-fluorophore distances.

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