Simultaneous quantitative depth mapping and extended depth of field for 4D microscopy through PSF engineering

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ABSTRACT

An extended depth of field (EDF) microscope that allows for quantitative axial positioning has been constructed. Past work has shown that EDF microscopy allows for features in varying planes to appear sharply focused simultaneously, however an inherent consequence of this is that depth information is lost. Here, a specifically engineered phase plate is used to create a point spread function (PSF) that contains both of the necessary attributes for extended depth of field and quantitative depth mapping. A two-camera solution is used to separate and capture the information for individualized post processing. The result is a microscope that can serve as an essential tool for full 3D, real-time imaging.

Keywords: 4D microscopy, PSF engineering, extended depth of field, quantitative depth ranging

1. INTRODUCTION

Extending a microscope’s depth of field allows for the investigation of a sample’s 3D structure. This is especially important in biological and medical research conducted with high numerical aperture objectives that have a very shallow depth of field. A number of methods are in use for extending the depth of field, such as confocal and wide field deconvolution\(^1,2\), but they suffer from slow acquisition speed, a high computational cost and expensive equipment. These techniques often require a lengthy multi-focus scan that prevents their use in live-cell imaging. Recent work has shown that these problems can be overcome with the use of point spread engineering and fast imaging processing techniques to create an extended depth of field (EDF) microscope for real time imaging\(^3-7\).

This approach to EDF microscopy engineers the PSF so that it becomes focus invariant over a range of up to 10 times longer than the original depth of field. This is done by simply inserting a specifically-designed phase mask into the back aperture of the microscope objective. The engineered PSF is then restored to a diffraction-limited spot through the use of a deconvolution filter. The PSF engineering approach to extended depth of field microscopy allows for real-time imaging since it only requires recording a single frame. Through the use of neural networks, nonlinear filters can be designed to faithfully reconstruct the signal while eliminating background noise and processing artifacts\(^7\). These features allow biological and medical researchers to more accurately observe the three-dimensional characteristics and dynamics of live cell processes. Figure 1 illustrates this PSF engineering approach. The top row shows a traditional PSF blurring with the defocus. A cubic phase mask\(^3,4\) creates the focus invariant PSF seen in the second row. A single deconvolution filter then restores all PSFs over the extended depth region back to diffraction-limited points.

While engineering the point spread function for focus invariance does allow for video rate EDF microscopy, it also inherently destroys any depth information associated with the blurring of the PSF. The observer can no longer distinguish the relative position of features within the field of view since all objects will appear to lie in the same plane. To overcome this limitation a new class of PSF has recently been developed which allows for extension of the depth of field and quantitative depth ranging simultaneously.
Figure 1. Illustration of the PSF engineering approach to EDF microscopy. The top row shows a traditional PSF blurring quickly with defocus. The middle row shows the PSF from the same objective with a cubic phase mask inserted in the back aperture. The PSF is now constant over the shown range of defocus. A deconvolution filter is then applied and the cubic PSFs are restored to diffraction limited, effectively extending the depth of field.

2. ASYMMETRIC POINT SPREAD FUNCTION

In order to extract depth information from EDF images a new type of PSF is required that exhibits both of the seemingly contradictory properties of focus invariance and changing through depth. However, this can be achieved through the use of a highly asymmetric point spread function, one that demonstrates surprisingly different properties on one side of best focus compared to the other. Specifically, we are introducing a PSF that is nearly constant in one direction starting from best focus, but creates rings that grow with defocus in the opposite direction from best focus. Figure 2 shows both sides of this asymmetric PSF for a 63x/1.4 NA oil immersion objective. Under normal operation the objective has a depth of field of approximately 0.5μm. The PSF remains focus invariant for upwards of 3μm on the left side of best focus. The rings start to form at 4.5μm to the right of best focus and remain above the noise floor for an additional 4.5μm. This asymmetry can be manipulated to create a microscope capable of EDF and quantitative depth mapping simultaneously.

Figure 2. Asymmetric PSF that allows simultaneously EDF and quantitative depth ranging. The top row shows left side of focus where the PSF is constant. The bottom shows a ringed PSF (to the right of best focus) that grows in radius with defocus. This asymmetric property can be exploited to incorporate quantitative depth mapping into an extended depth of field microscope.
The family of phase masks that create this unique point spread function can be seen in Fig. 3(a). They are circularly symmetric with a large singularity in the center where the largest phase delay takes place. The thickness of the phase mask then drops off radially. The radius of the PSF rings this mask produces can be easily calibrated to a distance from the best focus plane. They begin to form at 4.5μm away and are visible until approximately 9μm from best focus when using a 63x/1.4 NA objective. The calibration curve in Fig. 3(b) shows that the ring’s radius grows quadratically from best focus. The high correlation of this curve allows for the accurate mapping of the z position of points within an image.

![Circularly Symmetric Phase Mask for EDF with Depth Mapping](image)

(a)

![Calibration of PSF Radius to Depth](image)

(b)

Figure 3. (a) A sample profile of the family of phase masks that create the previously described asymmetric point spread function. (b) The calibration curve for the ring radius to distance from best focus.

Both properties necessary to extend a microscope depth of field have now been incorporated into a singular point spread function. The information is separated in space allowing it to be easily extracted for independent processing.

3. SIMULTANEOUS EXTENDED DEPTH OF FIELD AND DEPTH RANGING

A two-camera system adapted to a microscope is capable of simultaneously capturing both the extended depth of field and the depth ranging information for a given image. Figure 4 shows a basic diagram of the setup required. A phase plate is placed at the back aperture to modify the PSF, and a beam splitter is used to separate the light into two imaging paths. The first path is focused onto a CCD camera so that it observes the constant PSF. The second path has a slightly shifted focus so that the CCD views the ringed PSF that grows with depth. Using this technique both images can be captured simultaneously.

![Diagram of a two camera microscope capable of capturing both EDF and depth ranging information](image)
In order to show a proof of principle, a simple test object containing 0.1μm green fluorescent beads was imaged. Three beads lie in one plane while another two beads lie in a plane approximately 3μm deeper. The 63x/1.4 NA objective used only has a depth of field of 0.5μm so both planes are not visible simultaneously. Figure 5 shows the best focus plane for each grouping of beads and an intermediate plane half way in between using a traditional fluorescence microscope. If only one “best focus” image was recorded, the observer would not be able to tell there are two groups of beads present.

![Figure 5](image_url)

Figure 5. Images of 0.1μm beads separated in focus by 1.5μm. Using a high NA objective with a limited depth of field prevents beads lying in the separate planes from being imaged simultaneously.

The system shown in Fig. 4 is then used to capture both the extended depth image, see Fig. 6(a), and the depth ranging image, Fig. 6(b), simultaneously. In the extended depth of field image on the left all 5 fluorescent beads are now visible. They have been broadened slightly by a phase mask that creates the specifically engineered asymmetric PSF. The depth ranging image on the right shows a circle corresponding to each of the 5 beads on the left. The radius of each circle provides information on the z position of each bead. The images, acquired independently, can then be processed in parallel. The EDF image will be filtered using a nonlinear deconvolution filter, while depth ranging image will undergo a new processing algorithm that automatically identifies the presence of circles and determines their radii.

![Figure 6](image_url)

Figure 6. (a) Shows the extended depth of field image where all 5 fluorescent beads are simultaneously visible, although they are obscured by the modified PSF. (b) Shows rings of varying radii corresponding to each of the fluorescent beads. The size of the rings encodes the axial position of each bead.

### 3.1 Extended depth of field processing

The EDF image is processed using a nonlinear filter created with a neural network. The filter is iteratively optimized for a given objective and phase mask to produce the most faithful reconstruction of the original object. Since the filter is nonlinear it does not amplify high frequency noise from the CCD camera and the result is a high quality image with all
five beads in view simultaneously. This was achieved with a relatively low level of computational complexity, especially when compared to alternate methods such as wide field deconvolution. The fully processed EDF image can be seen in Fig. 7 where a sixth bead is visible even though it was barely above the noise floor in the encoded image. This information would have been lost if a standard linear deconvolution filter had been used. The asymmetric PSF was able to easily achieve a 6 times extension of the depth of field for a 63x/1.4 NA objective. This is comparable performance to the cubic point spread function previously used to extend the depth of field\textsuperscript{3,4}.

![Figure 7. Shows a nonlinearly processed EDF image of fluorescent beads. All objects are now clearly visible and sharply focused simultaneously. This technique only requires a single frame and can be easily executed in real time.](image)

### 3.2 Quantitative depth ranging processing

The algorithm for extracting the depth information centers around the circular Hough transform\textsuperscript{8}. The Hough transform is a technique commonly used in machine vision to identify circles. It can be most easily thought of geometrically where every point on a circle in the original image space is considered to be a center point in Hough space. A new circle of a given radius is then enscribed around each center point in Hough space and the overlapping portions are summed. If the given radius matches that of the original image space circle a maximum point will arise in Hough space. The exact size of each circle in our depth encoded image can be found by performing the Hough transform over the full range of possible radii and simply extracting the one which yields the maximum center value. Figure 8 shows the Hough transforms of the depth encoded image for two separate circle radii. Here the beads lying in the near plane produce a maxima near a radius of 51 pixels and beads in the far plane produce a maxima at a radius of 71 pixels. When the incorrect radius is used a doughnut shaped object appears instead of the singular point.

![Figure 8. Hough transforms of the depth encoded image for a radius 51 (left) and 71 (right) pixels. A maximum point (central dot) is seen when the radius used to perform the Hough transform closely matches that of the original circle.](image)
3.3 Three dimensional rendering

Using both the extended depth information and the quantitative depth mapping information a 3D rendering of the image can be created. The filtered EDF image yields the transverse position of all objects contained within the enlarged depth of field while the processed depth mapping image allows for the accurate positioning of the objects in the z-axis direction. Combining this information a full rendering can be created as shown in Fig. 9. Two views are provided that show the fully localized position of each bead to sub micron accuracy. Since the information used to generate this rendering was acquired simultaneously, at a single time point, this technique can readily achieve video-rate imaging.

![3D Rendering of 0.1 μm Fluorescent Beads](image1.png)

Figure 9. 3D rendering of all 5 fluorescent beads localized in x, y, and z. The two views show different angles along azimuth and demonstrate the fine separation of the individual particles.

4. CONCLUSIONS

Point spread function engineering has been used to create an extended depth of field microscopy system capable of simultaneous quantitative depth ranging, resulting in a 4D microscope (3D + real-time imaging). The system utilizes an asymmetric PSF that is focus-invariant on one side of best focus and forms rings that grow with defocus on the opposite side. The engineered PSF is implemented using a circularly symmetric phase mask at the back aperture of the objective. A two camera system is used to simultaneously capture EDF and depth encoded images. A nonlinear deconvolution filter restores the resolution of the extended depth image, while a circular Hough transform based algorithm extracts the depth map. This information is then combined to create a high resolution 3D reconstruction of a sample in real time. The system extended the depth of field by over 6 times and gave a quantitative depth map over 4.5μm for a 63x/1.4 NA objective.

Future work is focused on the imaging of live biological samples in real-time. Improvements to the EDF performance and accuracy of the depth map can be achieved through further design on the shape of the phase mask. Refinement of the processing algorithm will allow for the rendering of more complex images and processes. Ultimately this system will provide biomedical researchers with the capability to capture the most information possible about their samples in a real-time environment.

REFERENCES


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