Improved axial resolution of FINCH fluorescence microscopy when combined with spinning disk confocal microscopy

Nisan Siegel\(^1\) and Gary Brooker\(^1\)\(^2\)\(^*\)

\(^1\)Department of Biomedical Engineering, Johns Hopkins University, 9605 Medical Center Drive, Rockville, Maryland 20850 USA  
\(^2\)Microscopy Center, Johns Hopkins University Montgomery County Campus, Rockville, Maryland 20850 USA  
gbrooker@jhu.edu

Abstract: FINCH holographic fluorescence microscopy creates super-resolved images with enhanced depth of focus. Addition of a Nipkow disk real-time confocal image scanner is shown to reduce the FINCH depth of focus while improving transverse confocal resolution in a combined method called “CINCH”.

© 2014 Optical Society of America

OCIS codes: (090.1760) Computer holography; (100.6890) Three-dimensional image processing; (110.0180) Microscopy; (180.2520) Fluorescence microscopy; (180.6900) Three-dimensional microscopy; (090.1995) Digital holography.

References and links

1. J. Rosen and G. Brooker, “Digital spatially incoherent Fresnel holography,” Opt. Lett. 32(8), 912–914 (2007).
2. M. K. Kim, “Adaptive optics by incoherent digital holography,” Opt. Lett. 37(13), 2694–2696 (2012).
3. J. Rosen and G. Brooker, “Non-scanning motionless fluorescence three-dimensional holographic microscopy,” Nat. Photon. 2(3), 190–195 (2008).
4. G. Brooker, N. Siegel, V. Wang, and J. Rosen, “Optimal resolution in Fresnel incoherent correlation holographic fluorescence microscopy,” Opt. Express 19(6), 5047–5062 (2011).
5. J. Rosen, N. Siegel, and G. Brooker, “Theoretical and experimental demonstration of resolution beyond the Rayleigh limit by FINCH fluorescence microscopic imaging,” Opt. Express 19(27), 26249–26268 (2011).
6. P. Bouchal, J. Kapitán, R. Chmelík, and Z. Bouchal, “Point spread function and two-point resolution in Fresnel incoherent correlation holography,” Opt. Express 19(16), 15603–15620 (2011).
7. X. Lai, Y. Zhao, X. Lv, Z. Zhou, and S. Zeng, “Fluorescence holography with improved signal-to-noise ratio by near image plane recording,” Opt. Lett. 37(13), 2445–2447 (2012).
8. B. Katz, J. Rosen, R. Kelner, and G. Brooker, “Enhanced resolution and throughput of Fresnel incoherent correlation holography (FINCH) using dual diffractive lenses on a spatial light modulator (SLM),” Opt. Express 20(8), 9109–9121 (2012).
9. N. Siegel, J. Rosen, and G. Brooker, “Reconstruction of objects above and below the objective focal plane with dimensional fidelity by FINCH fluorescence microscopy,” Opt. Express 20(18), 19822–19835 (2012).
10. P. Bouchal and Z. Bouchal, “Wide-field common-path incoherent correlation microscopy with a perfect overlapping of interfering beams,” J. Eur. Opt. Soc. Rapid Publ. 8, 13011 (2013).
11. N. Siegel, J. Rosen, and G. Brooker, “Faithful reconstruction of digital holograms captured by FINCH using a Hamming window function in the Fresnel propagation,” Opt. Lett. 38(19), 3922–3925 (2013).
12. G. Brooker, N. Siegel, J. Rosen, N. Hashimoto, M. Kurithara, and A. Tanabe, “In-line FINCH super resolution digital holographic fluorescence microscopy using a high efficiency transmission liquid crystal GRIN lens,” Opt. Lett. 38(24), 5264–5267 (2013).
13. G. Brooker, S. McDonald, G. Adams, and J. Brooker, “Microscope attachment for high precision and efficient imaging,” US Patent 6,147,798A (2000).
14. M. Born and E. Wolf, Principles of Optics, 7th ed. (Cambridge University, 2009).
15. I. Yamaguchi and T. Zhang, “Phase-shifting digital holography,” Opt. Lett. 22(16), 1268–1270 (1997).
16. W. S. Rasband, “ImageJ,” NIH, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/ (1997–2014).
17. G. Brooker and B. Storrie, “3D Holographic and 2-photon super resolution microscopy,” http://www.nist.gov/public_affairs/releases/2010_johnshopkins.cfm.
18. R. Kelner, B. Katz, and J. Rosen, “Optical sectioning by confocal Fresnel incoherent correlation holography,” in Digital Holography and Three-Dimensional Imaging (Seattle, WA, July 13–17, 2014).
19. R. Kelner, B. Katz, and J. Rosen, “Optical sectioning using a digital Fresnel incoherent-holography-based confocal imaging system,” Optica 1(2), 70–74 (2014).
1. Introduction

Self-referenced holographic techniques [1–5] such as FINCH [1, 3–12] offer advantages over standard coherent holographic techniques, including the lack or requirement for use of a laser in the recording process, suitability for any illumination technique, and, for FINCH and similar techniques, inherent super-resolving capability for objects located away from the focal plane of the entry lens into the system. Thus, since the FINCH concept provides a platform for flexible and adaptable methods with the ability to offer high resolution 3D fluorescent images with little additional difficulty beyond standard imaging techniques, they are increasingly studied as techniques for improving microscopy performance. In particular, FINCH has been shown to exceed the Rayleigh limit in a microscope [5], offering high performance and focusable images from objects above and below the focal plane of the objective [9]. Recently, advances in computational techniques [11] and transmissive polarization-sensitive lenses [12] have further improved the performance of FINCH and show the potential for decreasing the exposure time and number of exposures required to create FINCH image stacks.

As the FINCH process recreates the 3D image field containing the 2D image that would be recorded by a traditional imaging method, the resulting refocused FINCH images taken under standard microscope configurations are analogous to widefield images, with the desired focused image as well as significant resolution of out-of-focus planes. In certain applications it would be desirable to reduce or eliminate those out-of-focus contributions while maintaining the inherent FINCH super-resolution advantage, resulting in a final high quality FINCH image of a single plane with super-resolution and low background. To that end we utilize a commercial confocal excitation/emission unit designed by one of us to create confocal images and confocal FINCH holograms of a biological sample at high magnification and with high NA objectives. For simplicity we call the confocal FINCH technique CINCH for Confocal Incoherent Holography. We compare these CINCH images to corresponding confocal and widefield images as well as to widefield FINCH holograms of the same samples and show improved background or axial contrast for the CINCH reconstructed images. Furthermore we show images taken of different planes in samples, with significant reductions in background and concurrent improvements in resolved/image quality for CINCH images. To the best of our knowledge these are the first FINCH-type holograms published for biological samples imaged with high-power (>40x), high-NA objectives.

2. Methods

The sample slides studied included fluorescent beads, pollen grains (Carolina B690) and a hematoxylin and eosin stained (H&E stain) human fundic stomach section (Carolina H7925). The fluorescent bead sample consisted of 1 μm Fluospheres (Invitrogen 8820, 540/560) dried from suspension onto a coverslip and microscope slide respectively. The coverslip was then mounted onto the microscope slide with optical cement (Thorlabs NOA65) with a 50 μm separation between the coverslip and slide. The FINCH transmission optical components and arrangement are substantially the same as those previously reported [12], with the exceptions that (i) they were configured as a inverted microscope, and (ii) instead of a relay system to project the back of the objective onto the GRIN/tube lens combination, a commercial aftermarket confocal head (CARV, Atto Biosciences) with a spinning Nipkow disk (Zeiss 452252) was used [13]. The confocal head is designed to be used as a 4f relay; the Nipkow disk is located at the internal focal plane of a relay consisting of a 200 mm “exit” lens (L2 in
Fig. 1. Schematic diagram of a microscope offering a combination of simultaneous classical and holographic imaging in widefield or confocal modes. Widefield mode includes classical widefield fluorescence and holographic FINCH, and confocal mode includes classical spinning disk confocal and holographic confocal CINCH. This configuration allows for simultaneous holographic and classical imaging of the same exact field of a specimen. The output of an infinity objective passes through a microscope tube lens (L1) which acts as one lens of a 4f relay terminated by lens L2. At the intermediate image plane of the 4f relay system a spinning Nipkow disk can be inserted for CINCH imaging to present the holography optical train with a single plane image. The area marked with dashed lines represents the commercial confocal head (CARV) used in this work. The fluorescence excitation light from an arc source (Photofluor II) passes through an excitation filter and is directed by a dichroic mirror through the disk and then excites the sample. The emission light passes back through the dichroic mirror and the emission filter through the L2 lens of the 4f relay. A polarizing beam splitter (PBS) directs the s polarized light through another tube lens (L3) to respectively record either a confocal or widefield image on camera 2 depending upon whether the spinning disk is in the beam path. The widefield FINCH or confocal CINCH holographic image is likewise imaged by camera 1 depending upon the spinning disk placement after the image beam passes through lens L4, active GRIN lens G1, inactive GRIN lens G2, liquid crystal phase shifter $\phi$ and output polarizing filter (Pol.).

Fig. 1) built-into the unit and an “entry” tube lens (L1 in Fig. 1). In turn, we positioned the entry tube lens with its own front focal plane at the back plane of the objective. The GRIN/glass lens combination that creates the hologram is located at the back focal plane of L2 and thus the confocal head takes the place of the 4f relay reported before [10, 12]. As shown in Fig. 1, the confocal unit received the excitation light from the lamp (Photofluor II illuminator, 89 North) and reflected it off a dichroic mirror in a microscope filter cube and then projected it through a Nipkow disk. A Nikon tube lens was placed with its image side focal plane located at the Nipkow disk and its objective side focal plane located at the back plane of the objective. The objectives used were Nikon manufacture, CFI 20x 0.75 NA and CFI 60x 1.4 NA, as well as 60x 1.49 NA TIRF. The objectives and sample stage were mounted in line with the rest of the optics. The emission light was passed through the Nikon tube lens and Nipkow disk, and from there through the dichroic filter cube and through a second lens (200 mm focal length) focused on the disk (completing a 4f relay with the lens L1) and to the final tube lens or holography optical train, both of which imaged onto Hamamatsu ORCA Flash 4 CMOS cameras. By use of the polarizing beam splitter immediately after the confocal head exit, the two cameras simultaneously record both widefield and FINCH, or confocal and CINCH images. This facilitates direct comparison on the exact same sample between each classical method (widefield or confocal microscopy) and its analogous incoherent holography method (FINCH or CINCH) at any object position. Furthermore, comparison between the confocal or widefield methods is possible by simply
moving the spinning disk into or out of the optical path respectively. The (incoherent) fluorescence emission light used to create the holograms was passed through a Semrock Cy3 4040C set, which has a 40 nm emission pass-band from 573 to 613 nm. If 40 nm is taken as the maximum bandwidth of the light (realizing that the emission light from the samples may have had a smaller bandwidth) this corresponds to a coherence length of at least \( \lambda_c^2/\Delta \lambda = 8.79 \) μm, where \( \lambda_c \) is the center wavelength of the emission and \( \Delta \lambda \) is the emission bandwidth [14]. The objectives were mounted in a Physik Instrumente piezoelectric z-stepping mount to enable precision control of the location of the sample or object relative to the objective focal plane. Widefield and confocal images as well as FINCH and CINCH holograms were recorded by the cameras.

Axial sequences, or “z-series,” of images were taken by stepping the piezo-mounted objective by various distances through the object, as described for each experiment. During the confocal and CINCH data acquisitions, the spinning disk was rotated at a speed sufficient to allow light from all transverse locations in the objective focal plane to pass through to the remainder of the system during each exposure, but slow enough to not introduce undue vibrations into the system.

Reconstructed images were created using the Fresnel propagation methods described previously [4, 5, 9, 11, 15]. In this work only one reconstructed image from each FINCH or CINCH hologram is used. This is termed the “FINCH image” or “CINCH image,” distinct from the FINCH or CINCH hologram. A simple object, for example a fluorescent bead, was used as a calibration. For each optical configuration, the calibration object was imaged at the objective focus with the regular fluorescence method and with FINCH or CINCH. A series of Fresnel propagated holographic reconstructions was generated with slightly different propagation parameters \( z_{rec} \). The best-focused reconstructed image in the propagation series was identified, and the corresponding \( z_{rec} \) was used to generate the FINCH or CINCH image from any FINCH or CINCH hologram captured with the same optical configuration.

3. Results and discussion

To examine the effects of applying the confocal method to FINCH holography, a sample object with two layers of 1 μm beads, separated by 50 μm, was stepped in 5 μm increments through the focal plane of a 20x Nikon objective. The results are summarized in Figs. 2 and 3, which depict three sample planes, denoted 1, 2 and 3, at relative depths of 0, 25 and 50 microns in the sample. Sample planes 1 and 3 contained the individual layers of beads, while sample plane 2 was equidistant between the layers. For each sample plane, Figs. 2 and 3 show (a-c) a raw FINCH/CINCH hologram (at one of the three phase shifts) as recorded on the camera, (d-f) a phase map calculated from the three recorded phase shifted holograms, (g-i) the reconstructed FINCH/CINCH image corresponding to the objective focal plane and (j-l) the comparative classical widefield or confocal image of the same plane. In Fig. 2 the comparison between widefield microscopy and FINCH shows that while FINCH faithfully reproduces a given plane of the object, light from other planes of the object form holograms as well, which are reconstructed during the Fresnel propagation process. This is shown in Figs. 2(a), 2(c), 2(d), and 2(f) depicting holograms recorded with one or the other of the bead planes at objective focus. In these images, the larger holograms and phase patterns are actually formed by the beads in the sample plane far away from the objective focus. In Figs. 2(b) and 2(e) it is shown that for an objective focal position equidistant in depth between the two object planes, the beads in both planes actually all produce similar holograms recorded by the camera. The FINCH image presented in Fig. 2(h) for sample plane 2 was calculated for the same depth as the FINCH images in Figs. 2(g) and 2(i) for sample planes 1 and 3, corresponding to the objective focal plane,
Fig. 2. Widefield fluorescence and FINCH imaging of a sample with two layers of 1 μm beads separated by ca. 50 μm, taken with a 20x Nikon objective. (a-c) The FINCH holograms are shown as log(intensity) to emphasize the recording at the camera plane of holograms from both bead planes. (d-f) Hologram phase maps, (g-i) FINCH images and (j-l) widefield images are displayed in a linear scale with intensity bars indicating the relative intensity of each image. Each column of images results from image capture with the designated sample plane at the focal plane of the objective. Sample plane 1 contains the top layer of beads, sample plane 3 contains the bottom layer and sample plane 2 is equidistant between the two layers. The FINCH phase images contain the depth dependent phase information derived from the FINCH holograms and the FINCH images show the complex FINCH holograms propagated to the best focal plane.

and is thus comparable to the corresponding widefield image for sample plane 2 shown in Fig. 2(k). However, the hologram in Figs. 2(b) and 2(e) from sample plane 2 – which originates from both bead layers – would all create focused FINCH images in the same reconstruction plane, mixing the two object planes, as indicated by their very similar phase maps. This is in accord with the curves published in Fig. 8 of [9], and illustrate the fact that for FINCH, in some circumstances it is required to ensure that all light used to create the holograms be from one side or the other of the objective focal plane.

In Fig. 3 it is shown that CINCH addresses this and uses the confocal method to eliminate light from planes away from the focus of the objective from creating holograms. In
Fig. 3. CINCH and confocal imaging of a sample with two layers of 1 μm beads separated by ca. 50 μm, taken with a 20x Nikon objective. (a–c) The CINCH holograms are shown as log(intensity). (d–f) Hologram phase maps, (g–i) CINCH images and (j–l) confocal images are displayed in a linear scale with intensity bars indicating the relative intensity of each image. Each column of images results from image capture with the designated sample plane at the focal plane of the objective. Sample plane 1 contains the top layer of beads, sample plane 3 contains the bottom layer and sample plane 2 is equidistant between the two layers. The CINCH phase images contain the depth dependent phase information derived from the CINCH holograms and the reconstructed CINCH images show the complex holograms propagated to the best focal plane.

The holograms in Figs. 3(a) and 3(d) taken of sample plane 1 at objective focus, only beads in that layer contribute light, while the beads in sample plane 3 (further away from the objective) contribute no light at all. The CINCH image in Fig. 3(g) shows only the sample plane 1 beads as does the confocal image in Fig. 3(j). In holograms in Figs. 3(b) and 3(e) taken with the objective at sample plane 2 equidistant between the two bead layers, there is no significant light from either plane in either the CINCH or confocal images in Figs. 3(h) and 3(k). When sample plane 3 was at objective focus (in Figs. 3(c) and 3(f)), the beads in sample plane 1 - closer to the objective - contributed no light at all to the CINCH image or confocal image in Figs. 3(i) and 3(l). Thus CINCH can be used to create unambiguous holograms that do not mix object planes.

To further examine the performance of CINCH in comparison to widefield and confocal microscopy and FINCH, the 1 μm beads were studied by all four methods without...
changing the transverse position of the sample using a 60x Nikon 1.49 NA TIRF objective. The higher magnification ensured that the system was capable of sectioning the target bead, which was larger than the PSF of the system in all dimensions. A single 1 μm bead was studied with all four methods, and a z-series was collected by stepping the sample through the objective focal plane in 200 nm steps. The widefield and confocal images at each step in the z-series were taken and used to create image stacks, as were the FINCH and CINCH images corresponding to the objective focal plane as described in the methods. These image stacks were analyzed by taking transverse profiles across the best-focused image and by taking axial profiles through the entire stack, as shown in Fig. 4. The results indicate that while all four methods produced similar results for the best-focused image, the axial profile of the CINCH z-series was a significantly better match for the confocal z-series than the FINCH z-series was for the widefield z-series. This result suggests that axial resolution and image quality is better for CINCH than FINCH.

To demonstrate the applicability of CINCH to biological samples, two biological samples were studied as well. Fluorescent pollen grains were imaged by all four methods using a Nikon 60x 1.4 NA CFI objective, while a H&E stained fundic stomach section was studied by confocal and CINCH methods using a Nikon 60x 1.49 NA TIRF objective. The design of the system with two cameras makes this exact comparison easy since the same
image field is simultaneously acquired for widefield and FINCH images or confocal and CINCH images. The comparison of the images of the pollen grains in Fig. 5 provides further insight into the advantages of CINCH over FINCH to obtain higher transverse resolution than classical imaging. The sample planes in these images were separated by 25 μm, each plane containing a focused pollen grain that is out of focus in the other plane. We refer to the pollen grain which lies partially in the objective focal plane as in-focus, even though parts of that pollen grain are certainly not focused, while the pollen grain that is very far from the focal plane is referred to as the out-of-focus pollen grain. While the widefield images (a) and (e) in Fig. 5 show blurred spots from the out-of-focus pollen grains, in the FINCH images (b) and (f) these out of focus pollen grains are relatively brighter and more featured, while the in-focus pollen grains show some contribution of light from other parts of the same pollen grain that are not quite at focus. In contrast, the confocal images in Figs. 5(c) and 5(g) have the...
Fig. 6. Confocal and CINCH images of a H&E Human Fundic Stomach section (top left and right respectively) taken using a 60x TIRF objective. Below each image is an intensity profile of the identical area from each image depicted by the red line. The CINCH image is the best plane of focus calculated from the Fresnel propagation. The confocal image is the image captured on the second camera during the capture of the CINCH holograms. The images without modification were opened in ImageJ [16] and the intensity profiles recorded. Images are 141 μm × 141 μm (confocal) and 145 μm × 145 μm (CINCH).

expected elimination of out-of-focus blur; while the corresponding CINCH images (d) and (h) do still show some blur from the out-of-focus pollen grains, the in-focus pollen grain images are nearly identical to the confocal images and show no contribution from other parts of the in-focus grain.

As shown in Fig. 6, the application of CINCH to a fundic stomach section shows the potential of this holographic method to produce better resolution images than classical confocal microscopy. At first glance the CINCH image appears similar to its confocal counterpart. However, careful perusal of both images reveals that the CINCH image reveals more in focus information, even though the CINCH image comes from the identical focal plane that created the confocal image. This is especially apparent when the bottom right portion of the image is examined. Notice that the confocal image is blurred but that the CINCH image reveals considerable detail. When a quantitative comparison of the two images was made by comparing intensity profiles of the same section from the two images it can be seen that image contrast and visibility in the CINCH image is considerably better than that of its corresponding confocal image, as the CINCH profiles return closer to baseline between
peaks and thus have less influence upon the next peak. This is consistent with the improved resolution previously reported for FINCH imaging.

4. Conclusions

As part of ongoing efforts to improve the image quality of FINCH and similar methods, we have investigated the application of confocal excitation/emission to FINCH, resulting in a method that we call CINCH. The new technique in which FINCH imaging was applied to a confocal slice achieves image quality for biological samples with high power, high NA objectives that improves transverse resolution beyond the limiting case for classical confocal microscopy. CINCH demonstrates what is possible with FINCH when the light from different object planes can be disambiguated – a system with transverse super-resolution in few exposures is achievable, accompanied by confocal axial resolution. It should be noted that the spinning disk confocal method is only one of a number of confocal methods that might be employed for this purpose. Other means of disambiguation are also possible and include laser scanning confocal microscopy as well as combining FINCH with a single image plane such as from two-photon confocal microscopy [17]. Complex deconvolution methods may provide similar benefits. A proposed SLM-based confocal FINCH [18,19] has recently appeared.

Acknowledgments

We thank Asma Azam for expert technical assistance. Research reported in this publication was supported by Celloptic, Inc., and the National Institutes of Health National Institute of General Medical Sciences Award Number U54GM105814 and the National Cancer Institute Award Number R44CA192299. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.