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CINCH (confocal incoherent correlation holography) super resolution fluorescence microscopy based upon FINCH (Fresnel incoherent correlation holography)

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Abstract

FINCH holographic fluorescence microscopy creates high resolution super-resolved images with enhanced depth of focus. The simple addition of a real-time Nipkow disk confocal image scanner in a conjugate plane of this incoherent holographic system is shown to reduce the depth of focus, and the combination of both techniques provides a simple way to enhance the axial resolution of FINCH in a combined method called "CINCH". An important feature of the combined system allows for the simultaneous real-time image capture of widefield and holographic images or confocal and confocal holographic images for ready comparison of each method on the exact same field of view. Additional GPU based complex deconvolution processing of the images further enhances resolution.

Keywords

Holography; FINCH; Fresnel incoherent correlation holography; fluorescence microscopy; confocal microscopy; Nipkow disk; super-resolution; deconvolution; GPU

1. Introduction

This laboratory has been focused on developing high resolution 3D microscopic imaging since its invention of a common path excitation/emission sectioning confocal microscope which utilized a Nipkow disk to create confocal image sections that could be assembled into 3D images of cells and tissues¹. That concept, called CARV, was initially commercialized, and subsequently has undergone additional commercial development². In an effort to create

3D fluorescent microscopic images without sectioning, a holographic approach was considered about a decade ago. We were initially inspired by the pioneering work of T.C. Poon and his colleagues^{3,4}, who for the first time, showed that microscopic fluorescence, which is incoherent, could be imaged by a holographic process. They called it “scanning holography” because a laser excitation interference pattern was scanned pointwise across the back aperture of a microscopic objective with subsequent correlation of the pointwise emission with each point of excitation. Our laboratory subsequently developed a common path self-referenced holographic technique, which we called FINCH^{5,6} for Fresnel Incoherent Correlation Holography. FINCH has certain advantages for microscopy over standard coherent holographic techniques since it: (i) can detect fluorescence, (ii) is non-scanning, (iii) can simultaneously record a hologram of an entire scene on a camera, (iv) is a single beam-path system, making it more stable with simplified alignment, and (v) does not require laser excitation of fluorescence. We and others⁷⁻¹⁵ have continued its development over the years. Thus, the FINCH concept provides a platform for flexible and adaptable methods with the ability to offer high resolution 3D fluorescence images with little additional difficulty beyond standard imaging techniques. In particular, FINCH has been shown to exceed the Rayleigh limit in a microscope⁸, offering high performance and focusable super-resolved images from objects above and below the focal plane of the objective⁹. Recently, advances in computational techniques¹⁵ and transmissive polarization-sensitive lenses¹⁶ have further improved the performance of FINCH and shown 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 images at many planes as well as significant contribution of light intensity from 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. Recently we have accomplished that goal by incorporating our commercial CARV confocal excitation/emission Nipkow disk unit with our newly developed high efficiency transmissive FINCH system to create an instrument that exceeds the overall XYZ resolution of FINCH and confocal microscopy¹⁷. In that paper¹⁷ and with a multi-dye specimen shown in this communication, we demonstrate the efficacy of that system to create high resolution confocal images and confocal FINCH holograms of a biological sample at high magnification and with high NA objectives, with exposure times of milliseconds to seconds depending upon the brightness of the sample. 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. Finally we demonstrate here

additional image improvement with further processing of the CINCH images by GPU powered complex deconvolution routines.

2. Methods

The FINCH transmission optical components and arrangement (see Figure 1) are substantially the same as those previously reported¹⁶, with the exceptions that (i) they were configured as an 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 used¹. 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 Figure 1) built into the unit and an “entry” tube lens (L1 in Figure 1). In turn, we positioned the entry tube lens (Nikon, 200 mm effective focal length) 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^{14,16}. As shown in Figure 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 20× 0.75 NA and CFI 60× 1.4 NA, as well as 60× 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 at 2K × 2K pixel resolution onto Hamamatsu ORCA Flash 4 sCMOS cameras for the bead, pollen and fundic stomach samples or PCO Edge 4.2 sCMOS cameras in the case of the HeLa cell imaging. 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 either a Semrock Cy3 4040C filter set, which has a 40 nm emission pass-band from 573 to 613 nm or Semrock YFP 2427B filter set, which has about a 30 nm emission pass-band from 525 nm to 555 nm. For the Cy3 set, 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 $\lambda_c^2 / \lambda = 8.79 \mu\text{m}$, where λ_c is the center wavelength of the emission and λ is the emission bandwidth¹⁹. For the YFP filter set the coherence length is about 9.72 μm . 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. It should be noted that our CINCH holographic confocal configuration differs from a recently proposed SLM based holographic confocal FINCH²⁰, since our method (i) utilizes all-transmission geometry and multiplexed analog pinholes rather than reflective SLMs and digital phase pinholes to obtain confocal FINCH images, and (ii) enables a ready comparison with either widefield or confocal images on the exact same field.

The sample slides studied included fluorescent beads, pollen grains (Carolina B690), a hematoxylin and eosin stained (H&E stain) human fundic stomach section (Carolina H7925) and HeLa cells dual immunolabeled for a Golgi protein and microtubules. 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. HeLa cells stably expressing a GFP-tagged Golgi apparatus protein¹⁸, (green, GalNAcT2-GFP) were also stained for microtubules with antibodies directed against alpha-tubulin (red, Cy3). Cells were grown for 2 days on glass coverslips, fixed with -20 degree C methanol for 4 minutes, antibody stained and mounted in a plastic polymer.

Axial sequences, or “z-series,” of images were taken by stepping the piezo-mounted objective by various distances through the object, as described in the discussion of the dual layer bead and pollen grain samples. 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. The complete CINCH system including real-time display of both cameras that displayed either the widefield and FINCH hologram images or confocal and CINCH hologram images and calculations were done using custom software written in National Instruments LabView. GPU deconvolution was done off-line.

Reconstructed images were created using the Fresnel propagation methods described previously^{5, 6, 7, 8, 15, 21}. In this work only one reconstructed image from each FINCH or CINCH hologram is used. This is termed the “FINCH image” or “CINCH image,” and is distinct from the FINCH or CINCH hologram. A simple object such as 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 20× Nikon objective. The results are summarized in Figures 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, Figures 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 Figure 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 Figure 2(a, c, d, 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 Figure 2(b, 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 Figure 2(h) for sample plane 2 was calculated for the same depth as the FINCH images in Figure 2(g, i) for sample planes 1 and 3, corresponding to the objective focal plane, and is thus comparable to the corresponding widefield image for sample plane 2 shown in Figure 2(k). However, the hologram Figure 2(b,e) from sample plane 2 – which originates from both bead layers – would create focused FINCH images of all the beads from both planes in the same reconstruction plane, mixing the two object planes, as indicated by their very similar phase maps. This is as calculated and shown in the curves published in Figure 8 of Reference 13, 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 Figure 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 the holograms in Figure 3(a, 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 Figure 3(g) shows only the sample plane 1 beads as does the confocal image in Figure 3(j). In holograms in Figure 3(b, 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 Figure 3(h, k). When sample plane 3 was at objective focus (in Figure 3(c, f)), the beads in sample plane 1 - closer to the objective - contributed no light at all to the CINCH image or confocal image in Figure 3(i, l). Thus CINCH can be used to create unambiguous holograms that do not mix object planes.

To demonstrate the applicability of CINCH to biological samples, three biological samples were studied. Fluorescent pollen grains were imaged by all four methods using a Nikon 60× 1.4 NA CFI objective, while an H&E stained fundic stomach section and HeLa cells were studied by confocal and CINCH methods using a Nikon 60× 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 Figure 4 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 Figure 4 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 Figure 4 (c) and (g) have the 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 Figure 5, 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 shows 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.

Additional demonstration of the utility of the CINCH technique is shown in Figure 6 for a two color fluorescence image. HeLa cells labeled with GFP expressed Golgi components¹⁸ and cy3 labeled microtubules were imaged. The darkness in the center of the cell is the nucleus. The Golgi apparatus is located juxtannuclearly in mammalian cells at or about the microtubule organizing center. The dark area towards the center of the cells is the nucleus. The results, shown in false color, indicate that FINCH is able to readily image two significantly different wavelengths without requiring resetting or re-optimization (aside from changing filters) when switching fluorescence bands, just as in standard widefield or confocal microscopy.

In the images in Figure 6, as well as the plots in Figure 7 and 8 showing the line profiles through highlighted features in Figure 6, it can be seen that CINCH shows improved contrast and resolution compared to confocal microscopy. However, in an effort to achieve the absolute best image, a deconvolution step was performed as well. Since the holographic

data collected by CINCH is processed in complex format, it is necessary to perform the deconvolution calculation in complex number space.

Building on the Microvolution (Danville, CA) technique of GPU-based deconvolution²² implemented as a plug-in to ImageJ²³, we developed a blind Richardson Lucy-type deconvolution routine to process the complex valued CINCH images. For the complex deconvolution, done here on each color channel as a two-dimensional deconvolution of a single focused reconstructed image, an empirical point spread function (PSF) was created as a starting point by acquiring holograms of subresolution beads, propagating the holograms to reconstruct the focused image of the subresolution beads, cropping out single beads, registering the beads to a constant reference, and finally averaging the beads. The PSF was used in complex format during the deconvolution, as were the reconstructed images of the tubulin and Golgi structures. We performed 7 deconvolution iterations for each channel which took less than 1 second for the deconvolution. The modulus of the final complex image is shown. Note that this deconvolution method is extensible to FINCH as well in both two-dimensional and three-dimensional deconvolution, with the substitution of an appropriate PSF. For the confocal images, we performed 7 iterations of a blind Richardson Lucy deconvolution algorithm. The starting PSFs were created by a theoretical Fraunhofer diffraction model²² with a numerical aperture of 1.49, refractive index of 1.515, backprojected pinhole radius of 778 nm, and emission wavelength of 525 nm or 590 nm for the golgi or microtubule images, respectively.

A comparison of the resulting deconvolved images at the bottom of Figure 6 to the original images at the top of the figure, as well as comparisons of the plots in Figures 7 and 8 show that the complex deconvolution method has similar effects on the CINCH image to those of the standard deconvolution on the confocal images. The background level is reduced, and there is increased contrast in the line profiles, indicating that the CINCH images are closer to their ideal potential after the deconvolution than they were before.

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. In the past, we have suggested the use of the spinning disk as shown here and other means of disambiguation such as laser scanning confocal microscopy as well as combining FINCH with a single image plane from two-photon confocal microscopy²⁴. In this communication, we now show the utility of complex deconvolution methods.

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Figure 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 ϕ and output polarizing filter (Pol.). From Reference 17.

Figure 2.

Widefield fluorescence and FINCH imaging of a sample with two layers of 1 μm beads separated by ca. 50 μm, taken with a 20× 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. From Reference 17.

Figure 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 $\log(\text{intensity})$. (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. From Reference 17.

Figure 4.

Two different object planes, separated by 25 μm , of a fluorescent pollen grain sample studied by four microscope methods with a 60 $\times$ Nikon CFI objective. The lobed pollen grain is in focus in plane 1 but out of focus in plane 2, while the spiked pollen grain is in focus in plane 2 but not in plane 1. From Reference 17.

Figure 5.

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 ImageJ²³ and the intensity profiles recorded. Images are $141\ \mu\text{m} \times 141\ \mu\text{m}$ (confocal) and $145\ \mu\text{m} \times 145\ \mu\text{m}$ (CINCH). From Reference 17.

Figure 6.

Top left and right images. Confocal and CINCH images (false color) of HeLa cells labeled with GFP (Golgi, green) and cy3 (tubulin, red). Note the increased contrast and definition of some finer features in both channels in the CINCH image. The dashed lines indicate where line profiles were taken of each labeled structure. The profiles are found in Figures 7 and 8. Bottom left and right images. Confocal and CINCH images (false color) of HeLa cells labeled with GFP (Golgi, green) and cy3 (tubulin, red) after blind deconvolution as described in the text. The dashed lines indicate where line profiles were taken of each labeled structure. The Golgi apparatus by electron microscopy has a width of about 300 nm and microtubules have a diameter of 24 nm. The image size for each confocal panel is $100 \times 90 \mu\text{m}$, and for each CINCH panel is $103 \times 93 \mu\text{m}$.

Figure 7.

Line profiles through the features indicated in Figure 6 in the red (tubulin) channel, as indicated by the vertical dashed lines in Figure 6. Comparisons can be made between the initial result of each imaging method and the deconvolved final image from each method. In each case the CINCH plot shows sharper features, and in the deconvolved plot lower relative background than the corresponding confocal image.

Figure 8.

Line profiles through the features indicated in Figure 6 in the green (Golgi) channel, as indicated by the horizontal dashed lines in Figure 6. Comparisons can be made between the initial result of each imaging method and the deconvolved final image from each method.