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Widefield fluorescence sectioning with HiLo microscopy

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***Abstract*— HiLo microscopy is a widefield fluorescence imaging technique that provides depth discrimination by combining two images, one with non-uniform illumination and one with uniform illumination. We discuss the theory of this technique and a variety of practical implementations in brain-tissue imaging and fluorescence endomicroscopy.**

I. Introduction

The main advantage of confocal microscopy over [1] conventional microscopy is that it provides depth discrimination within samples by rejecting out-of-focus light. The source of the depth discrimination in confocal microscopy is the combination of scanning point-illumination and pinhole-detection. However, the need for a scanning system introduces technical complications and cost issues which could be obviated in a wide-field setup. Alternative wide-field imaging modalities such as structured light illumination (SI) microscopy [2] and dynamic speckle illumination (DSI) microscopy [3-5] have been proposed and provide optical sectioning also through the idea of non-uniform illumination. We present a new imaging technique known as HiLo microscopy which utilizes both uniform and non-uniform illumination in order to produce an optically sectioned image [6,7]. HiLo microscopy is fast, robust and easy to implement. Moreover, it is generalizable to a variety of illumination and imaging configurations, and can be operated in both fluorescence and non-fluorescence modes.

II. HiLo microscopy principle

An optically sectioned HiLo image is constructed from the fusion of complementary in-focus high and low frequency image components. The high frequency components, which are inherently in focus, are derived by applying a high pass filter to the fluorescence image obtained with uniform illumination. The in-focus low frequency components are derived by evaluating the local contrast in the fluorescence image obtained with grid illumination. The fusion of the in-focus low resolution image with the complementary high resolution image (inherently in focus) results in a full resolution image that is axially resolved over all spatial frequencies within the microscope passband.

There are several possible techniques to evaluate the contrast of the structured-illumination image. Some of these include evaluating the image standard-deviation in local coarse-grained resolution areas [6]; performing single sideband-demodulation [7]; etc.. We will discuss the merits of these techniques, including some newer techniques that have been recently explored. It should be noted that at least three factors contribute to image contrast, namely illumination structure, object structure, and noise. In HiLo microscopy, we are only interested in an evaluation of contrast resulting from the first of these. We will therefore discuss various techniques to correct for object-structure and noise induced contrast.

Once a contrast evaluation has been performed on the structured-illumination image, this provides an "in-focus weighting factor" that can then be applied to the uniform illumination image, allowing the extraction of in-focus signal from the latter, albeit at low (coarse-grained) spatial resolution. In other words, we obtain an in-focus image that contains only low spatial frequencies. As noted above, the complementary high spatial frequencies are recovered by simply applying a complementary high-pass filter to the uniform illumination image. These high spatial frequencies are inherently in focus. A fusion of the high and low spatial frequency images described above then leads to an in-focus image that is axially resolved over all spatial frequencies, both high and low, with the imaging diffraction limit. This is the principle of HiLo microscopy.

III. RESULTS

To date, the principle of HiLo microscopy has been applied as an add-on to a standard widefield microscope with lamp illumination [6]. It has also been applied to fluorescence endomicroscopy using an imaging optical fiber bundle with a micro-objective [7]. In both cases, the images are acquired with a CCD camera that is synchronized with a mechanism that enables the rapid toggling between structured and uniform illumination. We have explored the use of various mechanisms ranging from a DMD (digital micro-mirror device) to a simple rotating diffuser plate. Quasi real-time HiLo imaging has been performed and demonstrated with tissue slices from GFP-labeled rat brains (HiLo microscopy) and from excised rat colon tissue labeled with acridine orange (HiLo endomicroscopy). Combined image acquisition and HiLo processing rates are upwards of

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5Hz. The imaging resolution is diffraction-limited in the case of HiLo microscopy, and limited by fiber-core sampling in the case of HiLo endomicroscopy. It should be emphasized that because the high-resolution information in a HiLo image is ultimately derived from only the uniform-illumination image (as opposed to being distributed being the uniform and structured illumination images), HiLo microscopy is very robust and insensitive to sample-motion induced artifacts.

IV. CONCLUSION

HiLo microscopy presents many significant advantages: 1) it is inexpensive; 2) it can be implemented as an add-on in any standard microscope; 3) it is robust and insensitive to defects in the structured-illumination pattern; 4) it is insensitive to motion-induced artifacts; 5) it is fast (in principle it is limited by half the camera rate); 6) it can be operated in both fluorescence and non-fluorescence modes.

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