Gradient field microscopy for label-free diagnosis of human biopsies

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Received 16 August 2012; revised 26 September 2012; accepted 30 September 2012; posted 2 October 2012 (Doc. ID 174441); published 30 October 2012

We introduce a label-free method for detecting high-grade prostatic intraepithelial neoplasia (HGPIN) in unstained biopsies. We image this condition based on the identification of basal cells in biopsies that otherwise would resemble prostate cancer by unassisted histologic examination. Gradient field microscopy (GFM) is used as a label-free imaging method which increases the contrast of a transparent sample by taking the first-order phase derivative that is very sensitive to rapid refractive index changes. GFM is able to image the basal cell layers in HGPIN biopsies because of their rapid refractive index changes at the periphery of small glandular structures.

OCIS codes: 170.0180, 170.1650, 170.4730, 170.6935.

1. Introduction

Due to their very low absorption and weak scattering, human tissues are mostly transparent under visible light spectrum and can be assumed as phase objects. The transmission function of a phase object is in the form of and thus shows no contrast when the object is imaged under a bright field microscope because all the structural information is in the phase of the field [1]. In order to solve the problem of imaging low-contrast objects, much microscopy development has been focused on finding different ways to increase the contrast. Exogenous contrast methods utilize chemical compounds, such as dyes, fluorophores, or nanoparticles to turn a phase object into an amplitude object. On the other hand, intrinsic contrast phase-sensitive methods use the information carried by the light passing through the phase object without turning the object into an amplitude object (see [2–5]).

As an important application of microscopy techniques, pathology practice has been largely dependent on staining methods to turn the tissue samples into amplitude objects [6]. The development of staining methods makes it possible not only to image tissues using a simple bright field microscope, but also to add specificity to different structures within a tissue sample [7]. Recently, quantitative phase imaging has emerged as a new way of examining pathology slides (see, e.g., Chap. 15 in [1]). For example, spatial light interference microscopy (SLIM) [8,9] can be used to detect and diagnose different diseases by quantitative phase in unstained slides.

In this paper, we show that imaging a phase derivative of the phase map has diagnosis value.

1559-128X/13/010A92-05$15.00/0

We present a method for label-free diagnosis of high-grade prostatic intraepithelial neoplasia (HGPIN) using gradient field microscopy (GFM), which is a *qualitative* phase method developed in our group [10,11].

2. Gradient Field Microscopy

A. Principle

Differential interference contrast (DIC) microscopy is one of the most popular noninvasive methods that increase the contrast of a transparent specimen [12]. At the core of this technique, there is the Nomarski prism, which splits the illumination beam into two slightly shifted beams that pass through the sample and get recombined at the detector. These two shifted beams generate an intensity image that is proportional to the first order derivative of the phase map, i.e., a component of the phase gradient, and thus yields very high contrast, especially at edges with rapid optical path length change. The idea of generating two slightly shifted beams like in DIC can be realized through Fourier filtering of an optical field. Such spatial filtering has proven its effectiveness in increasing the contrast through different applications such as phase contrast microscopy [13] and other, more recent techniques [14,15].

In our case, this beam shift is obtained by using the property that the Fourier transform of a sine function gives two delta functions separated by a distance inversely proportional to the period of the sine function:

$$FT[\sin(ak_y)U(k_x,k_y)] = \left[\frac{\delta(x,y-a)-\delta(x,y+a)}{2i}\right] \circledcirc U(x,y) \approx ia\frac{\partial U(x,y)}{\partial y}. \quad (1)$$

As shown in Eq. (1), a 2D multiplication of an optical field by a sine function gives in the Fourier plane the 2D convolution between the optical field in the image plane and two delta functions. In other words, applying a sine modulation in the Fourier plane splits the scattered optical field into two duplicated fields shifted by $2a$ at the image plane, where a is the inverse period of the sine modulation. Furthermore, for a small a, i.e., large period, the convolution can be approximated to a first-order differential, as shown in Eq. (1).

We applied the sinusoidal mask at the Fourier plane of a microscope image using an amplitude spatial light modulator (SLM). Due to the DC component, for a purely phase object, $U(x,y) = \exp[i\phi(x,y)]$. The intensity of the image (modulus square of the field), a first-order derivative of the phase of the object, is offset by a constant, namely,

$$I(x,y) = \left|U(x,y) + ia\frac{\partial U(x,y)}{\partial y}\right|^2 \approx 1 - 2a\frac{\partial \phi(x,y)}{\partial y}. \quad (2)$$

Notice that the result in Eq. (2) is identical to the result from regular DIC using Nomarski prisms. Obviously, the advantage of our approach is that it does not require specialized polarized illumination optics, and thus can operate with plastic (birefringent) specimen substrates [10]. Therefore, filtering in the Fourier plane of the image field with a sinusoidal amplitude mask gives an increase in the contrast by providing the first-order derivative of the phase of the object. It has also been demonstrated that using different filters such as parabolic or conic filters provide other modes of phase derivatives [10,11].

B. Gradient Field Microscopy

Figure 1 shows the GFM, which is an experimental setup for Fourier filtering using a sinusoidal amplitude mask. GFM is built as an additional module to a commercial bright field microscope, which itself is composed of a collector lens, aperture stop, condenser lens, objective, and tube lens. In order to obtain high spatial coherence in white light illumination, the aperture stop is closed down to the minimum size. From the image plane of the microscope, we place an optical module composed of two lenses to form a $4f$ system and a SLM. The first Fourier lens, L1, with the focal distance $f_1 = 75$ mm, is at a distance f_1 away from the image plane, and the SLM is located at a distance f_1 behind L1, which is the Fourier plane of the image field of the microscope. The second Fourier lens is at its focal distance, $f_2 = 150$ mm, away from the SLM and forms the modulated image at f_2 away, where the detector (Andor iXon$^+$ EMCCD) is located. In essence, the GFM module increases the contrast by imaging the first-order derivative of the phase of the sample and magnifies the image by a factor determined by the ratio f_2/f_1.

The SLM in our setup is a liquid crystal panel taken from an Epson Powerlite S5 commercial projector sandwiched between two cross polarizers. The top inset of Fig. 1 is projected to the amplitude SLM and the bottom is the profile of the sinusoidal modulation taken along the dashed line. The contrast ratio of this device, 400/1, provides sufficient attenuation at the region where the projected value should approach zero. Furthermore, the sine modulation period is calculated to be 7.8 mm (13 μm/pixel, 600 pixel/period) and yields at the image plane the spatial shift of $2\lambda f/a = 20$ μm between the two separated beams. The DC field is located between the two beams.

Along with the high contrast images, GFM has another advantage in its acquisition speed. Since the contrast is increased optically, there is no need of post-image processing. Thus, GFM naturally provides an ability to image in real-time and the acquisition speed is limited only by the frame rate of the detector. We used GFM to investigate unstained prostate biopsies characterized by HGPIN, as described below.

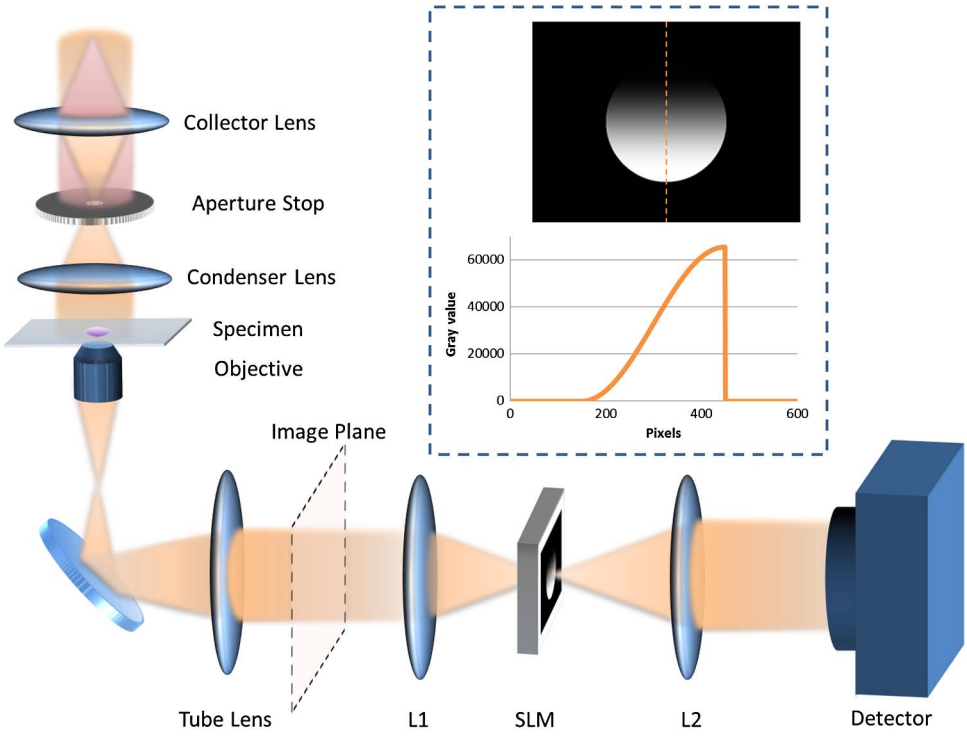

Fig. 1. (Color online) Schematic of the GFM setup. The components of a bright field microscope, Olympus 1 × 70, are shown in the figure (from the collector lens to the tube lens), along with the GFM module. Starting from the image plane of the bright field microscope, the Fourier lens L1 is located at one focal distance, 75 mm, and the SLM is located at another focal distance away from L1. The second Fourier lens L2 is located at one focal distance, 150 mm, away from SLM, and the detector is located at another focal distance behind L2. Inset shows the mask projected onto SLM and its vertical profile showing the sinusoidal function.

3. HGPIN Detection Using GFM

A. HGPIN Condition

HGPIN is a condition that mimics prostate cancer, with glands showing genetic and immunohistochemical changes associated with prostate cancer and tissue biopsies showing the presence of prominent nucleoli and Roman bridges in glands [16,17]. HGPIN is a lesion that is sometimes confused with prostate cancer in biopsies because it has atypical cells, large prominent nucleoli, but in contrast with cancer it has basal cells in the periphery of the glandular structures (Fig. 2). Because it mimics the morphology of prostate cancer biopsies, there is a need for investigation into HGPIN biopsies in order to correctly diagnose that they are in fact not malignant. Currently, cytokeratin 34BE12 and p63 immunohistochemistry markers are used to exclude the diagnosis of carcinoma by identifying basal cells [17]. However, immunohistochemistry delays diagnosis, usually by one day and frequently more.

B. HGPIN Detection Using GFM

The histologic presence of basal cells distinguishes HGPIN from prostate cancer. Since these cells are small in size, it is expected that they show rapid change in optical path length at the boundary and that they can be detected by GFM. With this idea, we imaged multiple unstained biopsies with HGPIN condition using GFM with a high magnification, high numerical aperture objective.

Figure 3(a) shows an image of a HGPIN biopsy taken under GFM with 100×/1.4 NA oil immersion objective on Olympus 1 × 70 microscope in bright field mode. Since this high magnification objective cannot image the whole biopsy at once, this image is produced by stitching 154 (14 × 11) images. Even though one core has to be imaged in many separate tiles, the fast acquisition speed of GFM allows high throughput in the measurement (10 min/biopsy

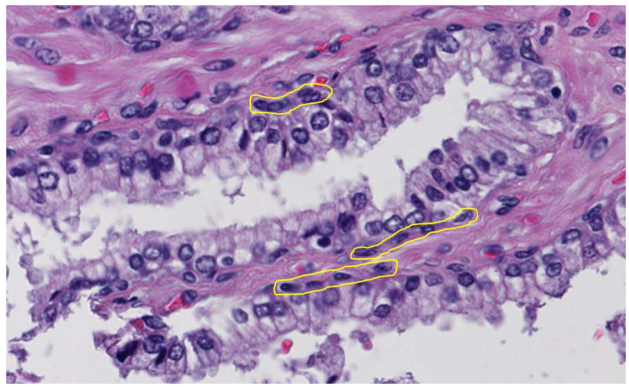

Fig. 2. (Color online) Hematoxylin and eosin stained slice of a tissue showing the basal cell layer. The flat layer of cells marked yellow between the epithelium and stroma area are the basal cells.

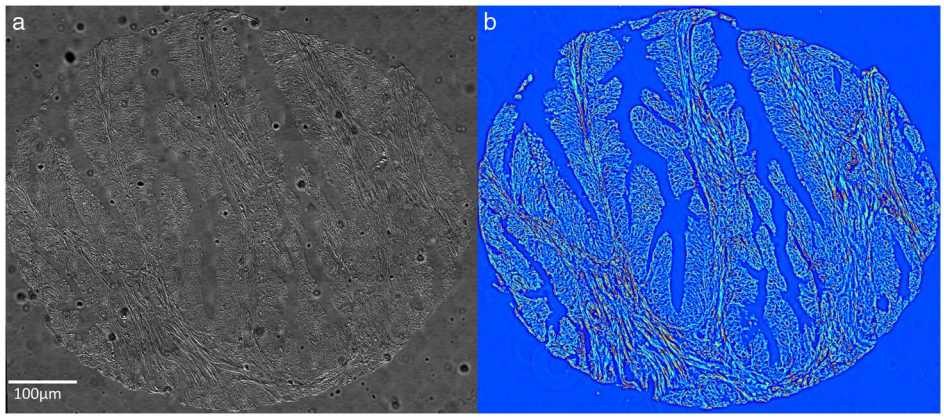

Fig. 3. (Color online) Images of HGPIN biopsies: (a) GFM image and (b) SLIM image.

assuming no error in stitching). In Fig. 3(b), a quantitative phase image of the same tissue taken using SLIM is shown. This image is also a stitch of 80 (10 × 8) images taken using a 40×/0.75 NA microscope objective. For each section (one field of view), it took 4 s to image the quantitative phase and for the whole tissue area, it took total of 9 min including the focusing time. Figure 4 shows 12 different HGPIN biopsies imaged by the same setup.

In order to show the high contrast at the edges more closely, the left column of Fig. 5 shows the GFM image of specific areas in HGPIN biopsies. Notice that the patchy basal cells are clearly seen between the epithelium and stroma area. Although they do not have any specific optical marker, it is possible to notice these cells from their boundaries, which shows high value in GFM, and also from their shape. To ensure this qualitative detection of basal cells done by eye based on the shape of the cells, the images are compared to the images of the same area taken using SLIM (the right column of Fig. 5), which is a quantitative phase imaging technique [8]. SLIM measures the quantitative refractive indices of a biopsy and uses it as a marker for different structures. It has been shown in Wang *et al.* [9] that the basal cells have low refractive indices in the quantitative phase image taken with SLIM and can be detected easily. Although there is no quantitative information in the GFM images, unlike in SLIM images, GFM shows very high sensitivity to edges, which gives the relevance in studying small and rapidly changing structures such as basal cells. We also have shown that the qualitative detection of basal cells using GFM matches well with the result from

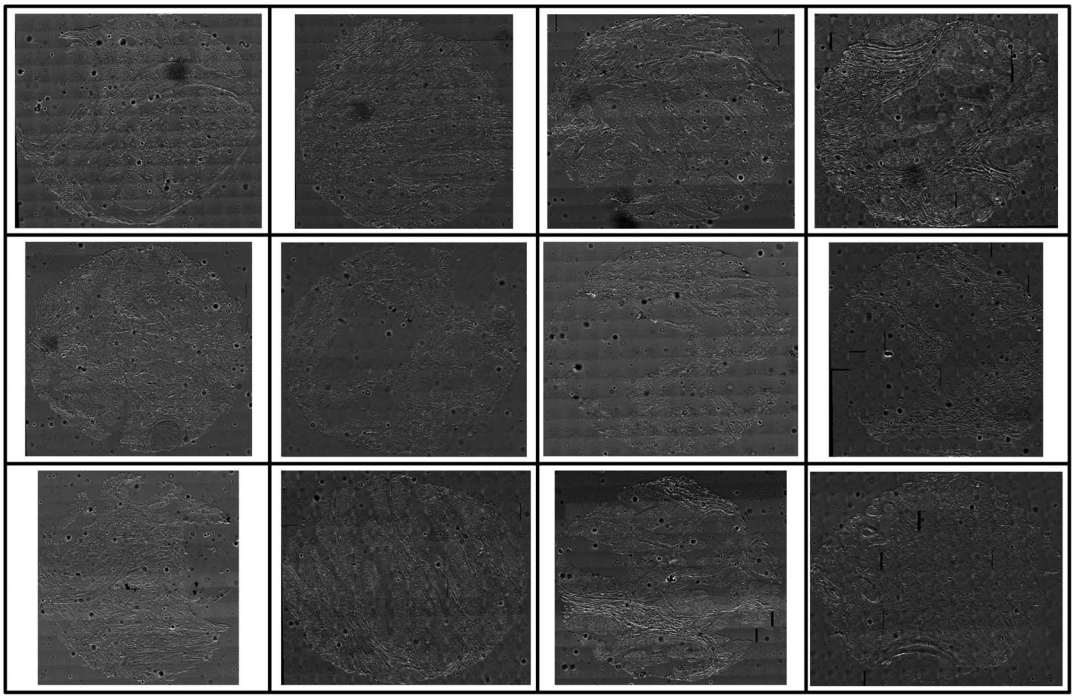

Fig. 4. Images of 12 different HGPIN biopsies taken under GFM with 100 × /1.4 NA oil-immersion objective. Each image is produced by stitching over 100 separate images.

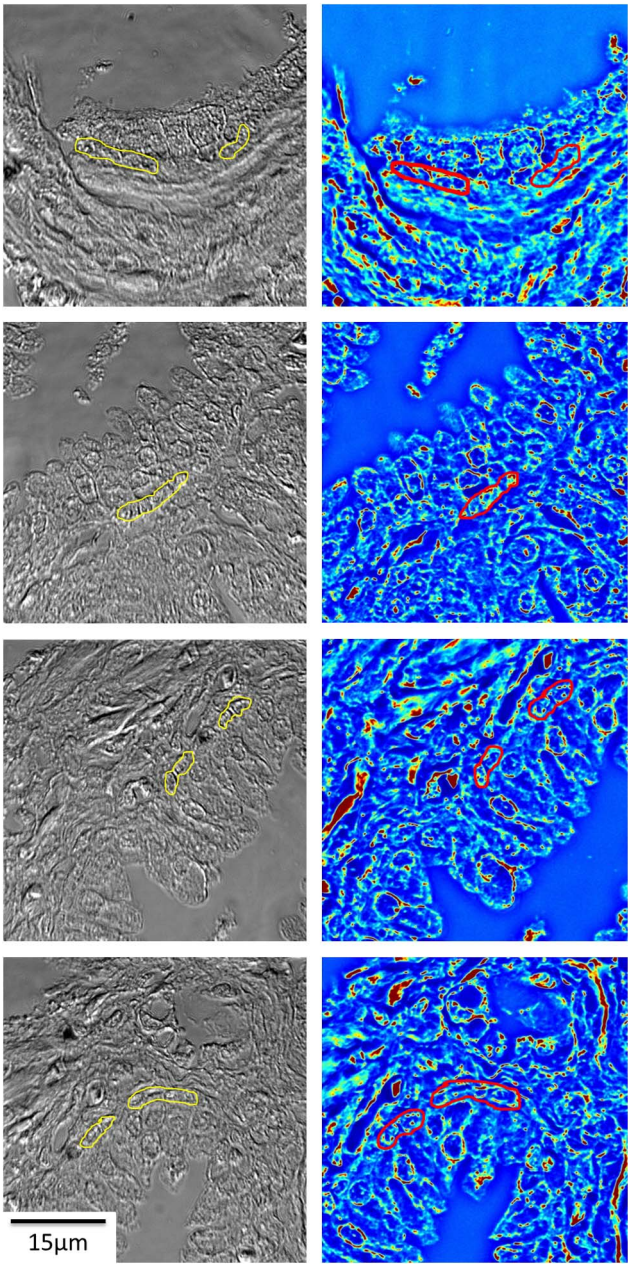

Fig. 5. (Color online) GFM (left column) and SLIM (right column) images of HGPIN biopsies showing the areas with basal cells. Images in the same row show the exact same region for comparison between GFM and SLIM. Basal cells are marked yellow in the GFM images and red in the SLIM images for clarity.

SLIM. Therefore, GFM can be used for detection of HGPIN condition in a fast (single shot) and label-free manner.

4. Summary

In this paper, we introduced a label-free method for detecting HGPIN condition in human tissue biopsies based on the presence of basal cell layers. GFM was used as a label-free intrinsic contrast method to increase the contrast in thin and transparent samples. This method provides an image that is proportional to the first-order differential of the phase of the object, thus providing high values at the edges where rapid refractive index change exists. Therefore, GFM is successful in detecting the small structure of basal cell layers, and thus identifying the HGPIN condition. Validation steps in an independent cohort of cases are in progress.

This research was supported by the National Science Foundation (grants CBET 08-46660 CAREER, CBET-1040462 MRI) and the National Cancer Institute (R21 CA147967-01). TK, SS, and GP acknowledge Dr. Jonathan Melamed for providing the HGPIN biopsy samples. For more information, visit http://light.ece.illinois.edu/.

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