Real-time brightfield, darkfield, and phase contrast imaging in a light-emitting diode array microscope

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Abstract. We demonstrate a single-camera imaging system that can simultaneously acquire brightfield, darkfield, and phase contrast images in real time. Our method uses computational illumination via a programmable light-emitting diode (LED) array at the source plane, providing flexible patterning of illumination angles. Brightfield, darkfield, and differential phase contrast images are obtained by changing the LED patterns, without any moving parts. Previous work with LED array illumination was only valid for static samples because the hardware speed was not fast enough to meet real-time acquisition and processing requirements. Here, we time multiplex patterns for each of the three contrast modes in order to image dynamic biological processes in all three contrast modes simultaneously. We demonstrate multicontrast operation at the maximum frame rate of our camera (50 Hz with 2160 × 2560 pixels). © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI.

1 Introduction

Brightfield, darkfield, and phase contrast are the most common label-free contrast modes used in optical microscopy. Brightfield imaging is most suitable for observing samples with strong absorption. Darkfield imaging provides good contrast for subresolution features, since it only captures high-angle scattered light. Phase contrast is used for unstained and transparent biological samples, allowing visualization of shape and density variations. There are several commercial choices for phase contrast [e.g., Zernike phase contrast or differential interference contrast (DIC)] and new methods which provide quantitative phase. Since each of brightfield, darkfield, and phase imaging provides complementary information about a sample, it is often desired to use multiple methods at once. However, in a traditional microscope, each contrast mode relies on a different optical hardware configuration, requiring inserts at the condenser aperture, polarization components, and/or specialized objectives. Here, we demonstrate a system which can achieve all three modes simultaneously by placing an LED array at the source plane and implementing simple postprocessing steps.

The LED array microscope is a new computational illumination platform in which a programmable source is combined with smart postprocessing algorithms to enable diverse imaging capabilities. An LED array is placed at the Fourier plane of the sample, replacing the original illumination unit of a commercial microscope (see Fig. 1). Each LED can be controlled individually to illuminate the sample from a unique angle, thus a set of LEDs on the array corresponds to a set of illumination angles. The brightfield imaging mode is achieved by illuminating with the central LEDs in the array, whereas darkfield uses LEDs corresponding to angles outside the imaging acceptance angle, set by the objective’s numerical aperture (NA). Recently, we have demonstrated that the phase contrast images can also be obtained in this system without any further hardware modifications. To do this, we employ asymmetric illumination differential phase contrast (DPC), in which one needs to only capture two images from complementary illumination angles. The resulting phase contrast image highlights shape and density variations along a single direction and is qualitatively similar to a DIC image. Three images taken under different illumination conditions are, therefore, sufficient for achieving all three brightfield, darkfield, and phase contrast modes. Five images can be used instead, if one wishes to capture the phase contrast along both orthogonal directions. We demonstrate here a hardware implementation that achieves such a multimodal contrast at high speed in a time-multiplexed fashion and show its use for imaging dynamic biological events.

Beyond the three contrast modes discussed in this paper, we note that the LED array microscope has recently been shown to be capable of enhanced resolution and three-dimensional (3-D) imaging, too, simply by choosing different illumination coding strategies. If one sequentially turns on each LED in the array, capturing an image for every illumination angle, the resulting dataset is four dimensional, akin to a light field. Thus, the data can be used to digitally refocus the image in postprocessing in order to synthesize 3-D intensity and phase contrast. Alternatively, if the object is thin, then the angular information can be used for computing large field-of-view, high-resolution gigapixel images via Fourier ptychography. While multiplexing achieves an order of magnitude reduction in the acquisition time and data requirements, these methods are still not suitable for real-time capture. Here, we focus on the three contrast modes that can be implemented in real time.
2 Multicontrast Imaging with an LED Array Source

To achieve brightfield, darkfield, and phase contrast modes, we capture time-interleaved images with three or five different LED patterns. The first pattern illuminates the darkfield LEDs of those which correspond to illumination angles larger than the maximum allowed by the imaging system. The radius of the circle defining the transition from brightfield to darkfield LEDs is set by the NA of the objective. When illuminating with darkfield LEDs, the only light that reaches the camera is that which has scattered from the sample, creating a darkfield image (see Fig. 2).

The second and third patterns are those of the left and right half circles, respectively, within the NA of the objective. Adding these two images together is equivalent to having taken an image with the full circle of LEDs, so it produces a brightfield image (with incoherence parameter $\sigma = 1$):

$$ I_{BF} = I_L + I_R. $$

(1)

We use the same two images to compute a phase contrast image, $I_{DPC}$, from the difference of the left and right side images according to the DPC method:

$$ I_{DPC} = \frac{I_L - I_R}{I_L + I_R}. $$

(2)

where $I_L$ denotes the intensity image captured with the left half of the brightfield LEDs on, and $I_R$ denotes the right half. Notice that the DPC image displays a shadowing effect along the horizontal direction because the patterns were split along the vertical direction. It is often useful to observe the phase contrast in both the horizontal and vertical directions, in which case we capture two more images with the top and bottom halves of the brightfield LEDs on. With the flexibility of the LED array patterning, any desired direction of asymmetry can be used to highlight features of interest.

Intuitively, the phase contrast obtained by DPC comes from the asymmetry of the illumination patterns. A single asymmetric illumination pattern alone gives good phase contrast in the captured images, but using both patterns together allows us to relate the information to quantitative phase. Consider a purely real sample (no phase variations), which will produce a symmetric distribution in the pupil plane. The images from complementary illumination patterns (e.g., left and right) will thus be identical for all real (absorption) effects. Thus, the contrast obtained by subtracting two images with either side of the source on comes only from phase information of the sample. In fact, the DPC image can be shown to be directly proportional to the derivative of phase along the direction of asymmetry.

Fig. 1 Our experimental setup uses a programmable LED array in the source plane of a microscope to pattern illumination angles. By time-multiplexing different LED patterns, real-time multicontrast imaging is achieved with a single camera and no moving parts.

Fig. 2 Our time-multiplexing scheme for multicontrast imaging in real time. (a) Five illumination patterns are displayed in a sequential loop on the LED array, while an image of the sample is captured for each. (b) The darkfield image is captured directly, while the brightfield and phase contrast images are calculated from sums and differences of the complementary halves of the brightfield LEDs, respectively. Here, we use both left-right and top-bottom phase contrast patterns; however, where only one direction of phase contrast is sufficient, only the first three patterns are necessary.
In x-ray, a similar phase derivative result is obtained by grating interferometers, which can be considered a parallelized split-detector DPC measurement on the imaging (rather than illumination) side. Darkfield can also be extracted and the derivative images can be integrated in order to recover the quantitative phase.

The ability to separate the phase from absorption information should be considered a significant advantage of DPC over phase contrast methods such as Zernike phase contrast, DIC, Hoffman modulation contrast, and Dodt contrast. The images produced look qualitatively similar to DIC, but contain no absorption effects. Since the illumination is partially coherent, the optical sectioning capabilities and lateral resolution of DPC are better than that of coherent techniques (interferometry) and the results are free from speckle noise. In addition, DPC does not rely on polarization optics, so the image will not suffer polarization artifacts for birefringent samples (e.g., muscle tissues and collagen fibers). DPC images can also be created without any modifications to the optical system after the sample (i.e., no specialized objectives or inserts). This simplicity of implementation has enabled its integration in real-time endoscopic imaging for in vivo studies.

3 Experiments

The experimental setup is an inverted Nikon TE300 microscope (Melville, New York) (shown in Fig. 1), where the illumination unit has been replaced with an LED array (32 × 32, 4-mm spacing) at 60 mm above the sample. The LED array subtends angles up to NA 0.73, ensuring that we can achieve darkfield illumination for most 40× and lower objectives. Note that the set of LEDs which make up the darkfield area will change with the objective NA, so the size of the brightfield circle should change based on the objective being used. Each LED is controlled individually in 8-bit grayscale by an Arduino (Italy) controller. An sCMOS camera (PCO.edge 5.5) is placed at the front port of the microscope for image collection and data are transferred to the computer via a CameraLink interface. The camera is synchronized with the LED array by the same controller.

To implement the time multiplexing, the five (or three) patterns are displayed on the LED array in a continuous loop while collecting images for each pattern. Since the LED pattern transfer time (~320 μs) is generally less than the exposure time required (e.g., 7 ms at 100 Hz), the maximum speed is limited by the camera frame rate. The captured images are processed in real time according to the simple operations in Eqs. (1) and (2) to obtain the multicontrast images (illustrated in Fig. 2). Results are regrouped to generate videos for each contrast mode.

Experimental results using HeLa samples are shown in Fig. 3(a). Darkfield imaging provides contrast to the cell membranes and boundaries can be visualized easily, which is helpful for cell counting in densely cultured plates. For cell morphology studies, where the shape or volume of the cell is important, phase contrast will be the most suitable tool. For example, some epigenetic modulators like Trichostatin A can change the epigenetic state of chromosomes and cause the cell morphology to change from a round to an elongated shape. As another example, multicontrast imaging of model plant Arabidopsis can clearly trace nutrient transportation tubes in the root [see Fig. 3(b)]. The transport of dark material in these root structures can be studied easily by this microscopy system, since the vessel and transportation particles can be visualized by darkfield and brightfield, respectively. Note that the root hair structure at the white arrow is clearly observed in the darkfield, and the central vessel of root at the black arrow is clearly resolved in the brightfield. The phase contrast images in horizontal and vertical directions also selectively enhance the contrast along those dimensions, rendering better contrast in the radial vessel axes.

A key application of our method will be for live unstained samples, demonstrated in Fig. 4 for Caenorhabditis elegans wild-type samples on nematode growth media petri dish substrate. The fast-moving and nearly transparent worms normally cannot be simultaneously tracked in different imaging modes. Using our LED array method, we achieve four-mode imaging at 50 Hz across all modes with 2160 × 2560 pixels in each image and a 16-bit dynamic range. A few sample frames of the resulting videos are shown in Fig. 4. Since these images were taken through the substrate, the phase contrast images highlight the small indentations left behind by the worms. The results are best appreciated by viewing Video 1.

Fig. 3 (a) Experimental results for HeLa cell samples imaged with a 20×, NA 0.4 objective in brightfield, darkfield, and Top-Bottom (T-B Phase) and Left-Right (L-R Phase) phase contrasts. (b) Experimental results for an Arabidopsis sample imaged with a 10×, NA 0.25 objective. All data were captured within 200 ms.
4 Conclusion

We have demonstrated the ability to capture multicontrast microscopy in real time for dynamic biological samples. Our results enable one to simultaneously visualize and compare images in brightfield, darkfield, and two directions of phase contrast. We demonstrated four modes of capture at 50 Hz with 2160 × 2560 pixels and a 16-bit dynamic range, with speed being limited only by the camera frame rate. For faster operation, one could use only three LED patterns (one direction of phase contrast) or trade off the pixel resolution or dynamic range for speed, up to a hardware limit of 500 Hz. Potential biological applications include live cell imaging studies, as well as small organisms such as *C. elegans* and *Drosophila* embryos. Unstained transparent samples are easily visualized in the phase contrast mode, while subresolution features (e.g., organelles and vesicles) show good contrast in darkfield. Due to the simplicity of the LED array microscope setup and the diverse range of imaging capabilities it enables, we expect that the LED array illuminator will find widespread use in a variety of applications.

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References


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