Shadow Technique Algorithm (STA) Sheds a New Light on Differential Interference Contrast (DIC) Microscopy

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Abstract

Diversity of biological samples is still partially considered by conventional Differential Interference Contrast (DIC) microscopy approaches. Here we propose a new algorithm (developed as an ImageJ macro), the STA (Shadow Technique Algorithm), whose originality relies in the 3D/4D visualization of a large range of biological objects, from bacteria, vegetal tissues to living cells in culture. This new approach does not need extensive calculations, systems modifications or in-depth knowledge of acquisition optics. STA, providing 3D DIC reconstruction every hundredth of ms, can be applied to dissect various cellular phenomena. In addition, we propose different methods of graphic representations, which unable to enlighten the specificities of each category of questioning. Specifically we here addressed: i) tissue imaging, ii) cell cycle and cell death imaging iii) vesicle tracking.

Keywords: Differential interference contrast; Shadow technique algorithm; Image analysis; ImageJ; Tracking; Cell cycle; 3D reconstruction

Introduction

Nomarski Differential Interference Contrast (DIC) is a non-invasive photonic microscopy method to gather structural properties at the cellular and sub-cellular levels [1]. In such context, living cells are not damaged, enabling the observation of dynamic processes both in vitro and in tissues. From the early fifties, DIC has provided nuances of grey, to detect small structures and vesicles [2,3]. Thus, one shall keep in mind that DIC is intrinsically qualitative, reflecting a difference in grey level. To do so, DIC's images result in a false sense of depth. Indeed, images are produced by interference between a reference and a spatially-sheared light wave, resulting in a differential image having directional contrast, according to the direction of the shear.

DIC microscopy was extensively used when considering objects in 2D, while thick objects have been more difficult to assess. Comparing to fluorescence, which is widely used for 3D and benefits from a black background and grey levels reflecting the intensity of the signal, DIC provides images of structures boundaries exhibiting either bright or dark intensities, while the remainder of the image has an intermediate grey level. Thus, standard image processing methods for DIC image segmentation, such as "thresholding" or edge detection, cannot be employed on DIC's images and limit 3D reconstruction (Figure 1) [4,5].

Several efforts have been made to develop 3D representation of DIC image series. They can be classified following two main approaches, qualitative or quantitative. The quantitative method needs precise knowledge of the shear angle of the DIC prism. They may be used or restriction to specific biological objects [7-10]. All together, these considerations have led users to consider DIC as a tough and rough tool for biologist, who are the main potential users for such methods.

In this article, we propose the Shadow Technique Algorithm (STA), a novel and user-friendly algorithm enabling the 3 and 4D visualization of DIC that overcomes the above-mentioned limitations (Figure 1). This method has been applied to represent biological sample ranging from thin and low-contrasted bacteria to thick and dense root slices. We also provide comprehensive methods to extract biological information and to represent sample morphologies.

Material and Methods

Caulobacter crescentus bacteria were grown to mid-log phase in PYE (Peptone-Yeast Extract) medium at 30°C [11]. For fixation,
bacteria were centrifuged and placed in 4% paraformaldehyde for 15 min at room temperature followed by 30 min on ice. After washing and resuspension in PBS, 2 µL of suspension were deposited on microscope slides coated with agar pads (1% agar in PBS).

**MDA cells**: Human MDA-MB-231 cells were cultured in Dulbecco’s modified Eagle's medium (Invitrogen, Life Technologies, Saint Aubin, France) supplemented with 10% fetal bovine serum and 50 ml gentamycin. 24 hr before observation, cell were seeded on a POC (Perfusion Open and Closed) chamber system (PeCon GmbH) and medium was replaced by Leibovitz’s L-15 Medium without phenol red (Sigma-Aldrich Co. LLC) just before observation.

Convallaria root slice is a reference sample kindly provided by Leica Microsystems GmbH.

**Image acquisition**

Experiments were carried out with a Leica AF6000 LX microscope (Leica Microsystems GmbH) equipped with a HCX PL APO CS 100X/1.40 DIC (Oil) objective and an Andor iXon DU-885 EM-CCD camera (Andor Technology Ltd). For live MDA acquisitions, the sample temperature was set to 37°C using a PeCon incubator (PeCon GmbH).

**Image processing**

**STA**: For details about STA, please see results, and Figure 2.

To use STA:
- Copy the text available as supplementary data in Fiji’s macro editor (Plugin/New/Macro).
- Select macro language in the “language” tab
- Optimize the initialization parameters if needed, as explained in the green header
- Open your image or stack
- Run the macro.

**Depth color coding**: We modified the “temporal color code” plugin developed by Kota Miura (Centre for Molecular and Cellular Imaging, EMBL, Heidelberg, Germany) to apply a color code on each image depending of its depth. Instead of applying a Maximum Intensity Projection of the stack, we choose to use the ImageJ 3D viewer, and thus obtained a 3D reconstruction of cells with and easily readable depth scale, as shown in Figure 3. The plugin we developed is available upon request.

**Combined volume and slice view**: To compare information usually obtained through 2D DIC imaging and 3D reconstruction along time, we used a mixed representation of the data in Figure 4. First, we performed a 4D reconstruction with high threshold applied on the 4D DIC series processed with STA and color coded it in red.
We then applied on the bottom of the bounding box the best focused unprocessed DIC image. Object correspondence between DIC and STA-reconstructed-DIC can thus be achieved by translation, as seen for vesicles located at the edge of the cell in Figure 4C.

**Particle tracking:** To illustrate our capacity to follow objects in the 4D, we used the trackmate plugin for Fiji as described in ref. [12]. We choose high threshold to segment vesicles only. Vesicles are then represented as pink spheres while their trajectories are shown as colored line, with colors temperature reflecting their velocity.

**Results and Discussion**

**STA method**

The method has been developed for Fiji [13], a distribution of ImageJ [14] for biological-image analysis. Original stack is duplicated
4 times. On each stacks, either a $3 \times 3$ north/south/east/west filter is applied (see Figure 2 for filters matrixes) producing a shadow effect on each image. The ImageJ “substract background” function is then applied, using the “rolling ball” algorithm [15], to substract local background values from the image. The 4 resulting images are then added up and processed by Contrast Limited Adaptive Histogram Equalization (CLAHE, [16]). 3/4D representations are then obtained on thresholded images either through Fiji’s “3D Viewer” or “volume viewer” plugin. STA is available has an ImageJ macro.

**Biological applications**

We choose different representative biological samples to illustrate the information that can be extracted by STA: (i) thin and delimited objects such as fixed bacteria, (ii) thick and structured object like section of complex architecture of *Convalaria* root and (iii) eukaryotic livings cells, such as MDA, to illustrate cellular deformation and intracellular dynamics. These objects would not have been easily and exhaustively considered by conventional DIC approaches.

Figure 1, with bacteria imaging, presents the typical problem encountered after direct 3D DIC reconstruction: object deformation and artifact appearance. After STA, bacteria morphology is faithfully pictured, preserving cell shape, size and curvature. It is thus possible to compare bacteria morphologies whatever their 3D orientation.

In Figure 3A and 3B, we represented the 3D reconstruction of a *Convalaria* root slice. As observed on such structured object, direct reconstruction was possible with optimized threshold and transparency adjustment. However, while we get a global idea of the biological object, cell walls and vacuoles were not realistically reconstructed (A,A’). After STA, the reconstruction was more consistent with reality, based on previous knowledge we had from fluorescence images. Indeed, the different cells walls were defined on the whole width of the stacks and the vacuole shape was preserved.

On C and D, the same approach was applied to MDA cells. In this case, the contrast was low, and an appropriate threshold could not be found directly from the DIC series (C). After STA, one may clearly visualize both thin plated cells (partial membranes of different cells are visible in blue-purple) and big round cells (coded from blue to red). This visualization could then allow following different cell processes like mitosis, apoptosis or necrosis which is not feasible using 2D DIC imaging.

We thus extended our approach to 4D imaging (Figure 4). Half of an MDA cell was represented, within which vesicles were easily observed. However, 2D DIC images allowed monitoring only vesicles at the focal plane of an image. It was thus well adapted to the edge of the membrane, which can be considered flat at the optical scale, but we failed to image vesicles on top of the cell. This was exemplified in panel C, a cropped portion of the initial cell over time. The vesicle highlighted by the white arrow was visible from the first time series on the 3D reconstruction, while it appeared in the focal plane of the DIC image only at the fourth time point (orange arrow). STA thus allowed 4D tracking of sub-cellular dynamic as illustrated in D and E.

**Conclusions**

**Amenability of STA**

We considered several levels of complexity within a biological object: thickness, inner architecture, cellular deformations and intracellular movements of organelles (Figures 3 and 4). Many observations cannot be gathered with conventional DIC use. More complex and time-consuming methods can be used, such as Hilbert transform, rotational diversity, and iterative line integration. Nevertheless, the STA enabled to address these objects, in a user-friendly manner and without requesting either microscopes adaptations or modifications.

In addition, we proposed different methods of graphic representations. Depth scale color coding in Figure 3 provided a glance the relief of membranes: healthy cells plated were visualized in blue-green whereas dying cells were highlighted in red (Figure 3D). Similarly, dividing cells, which are spherical as well, could be easily followed. Thus, morphology of cells can be monitored, with respect of all their aspects of life and death. Regarding organelles tracking, we proposed a mixed representation, merging classical 2D DIC images with 3D volume reconstruction (Figure 4). Such combination provided the familiarity of DIC images with the complementary information of 3D. Furthermore, it can be combined to particles tracking.

In an era that crowns quantitative approaches, still DIC manages to provide valuable and precise qualitative observations through STA. One shall also note that the observations gathered are sufficient to address biological phenomena in their complexity, with an extreme time-resolution without the limits of photo bleaching usually encountered in these contexts. Indeed, in our hands high-resolution images (1000 × 1000 pixels) were gathered every 3 ms and complete stack around 150 ms. Such fast acquisitions, with precise reconstruction of complex structures, provided the opportunity to monitor cellular deformation like the one occurring during cell death (Figure 3) or vesicles movements (Figure 4). Far from remaining an old-grand pa’s technique, DIC still provide, through the Shadow Technique Algorithm, a fancy tool for numerous biological applications.

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